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Molecular Detection of Enteroviruses in Cell and Tissue Samples

Implications in Type 1 Diabetes

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ACADEMIC DISSERTATION
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ABSTRACT

Type 1 diabetes (T1D) is a disease with no cure or prevention. In T1D, the insulin-producing pancreatic beta cells are impaired or lost due to a chronic autoimmune process, leading to a need for a life-long insulin treatment. Genetic predisposition determines the susceptibility to develop T1D but exogenous factors also contribute to pathogenesis. For example, numerous epidemiological studies have connected enterovirus infections to T1D. The aim of this study was to develop and evaluate methods for the detection of enteroviruses in cell and tissue samples. Furthermore, the aim was to employ these methods to assess the possible role of enteroviruses in the process leading to T1D by analyzing tissue samples from organ donors with T1D or T1D-related autoantibodies.

A commercially available *in situ* hybridization (ISH) technology was applied for the detection of enterovirus genome in formalin-fixed paraffin-embedded (FFPE) tissue sections. Enterovirus-specific probes that recognize selected regions from the viral genome were designed using advanced bioinformatics tools and validated in samples from virus-infected cell cultures and mice. The results showed that the ISH method enabled detection of enteroviruses in both human cells and mouse tissues infected with enteroviruses. In addition, the probes could be designed and optimized to bind either a single enterovirus type or alternatively a wide range of different enterovirus types.

Comparison of different enterovirus detection techniques in frozen and FFPE human cells containing different amounts of enterovirus-infected cells showed that RT-qPCR was the most sensitive method in frozen samples giving a positive signal in the most diluted sample (10^{-8}), followed by proteomics (10^{-7}). Immunohistochemistry (IHC) with a commercial enterovirus VP1 antibody detected enterovirus in FFPE samples at dilution 10^{-6}, and ISH at dilution 10^{-4}.

To assess enterovirus presence, the most sensitive methods were then employed to analyze a unique set of samples collected from cadaver organ donors in the Juvenile Diabetes Research Foundation (JDRF) funded Network for Pancreatic Organ Donors with diabetes nPOD. To assess enterovirus positivity, FFPE pancreas, spleen,

duodenum and pancreatic lymph node samples from T1D donors, donors positive for T1D -associated autoantibodies (AAb+) and non-diabetic control donors were immunostained using a commercial enterovirus VP1 antibody. As a result, enterovirus protein was found in tissues of T1D donors more frequently compared to control donors; in pancreas 70 % vs. 33 % (only insulin-positive organs included), in duodenum 40 % vs. 14 %, and in spleen 40 % vs. 26 %. Pancreatic lymph nodes were only available from a few T1D (N=9) and AAb+ (N=1) donors, and 56 % and 100 % of these were enterovirus-positive, respectively. When RT-qPCR was applied to detect enterovirus genome in frozen tissue samples, viral RNA was detected in all organs, except in the duodenum, but less frequently than viral protein. When enterovirus positive pancreata were studied with markers of antiviral response (class I HLA, MxA and PKR), it was observed that in T1D donors, the enterovirus positive islets expressed strongly class I HLA molecules and MxA. However, such HLA hyperexpression or MxA upregulation was not seen in enterovirus positive islets in control donors. This finding suggests that virus-induced cytokine responses may differ between the groups.

In conclusion, an enterovirus-specific *in situ* hybridization method was developed for fixed cell and tissue samples. This method was tailored by probe design to detect either a specific enterovirus type or a wide range of different enteroviruses. However, the method did not reach the sensitivity needed for human tissue samples with low viral titers. Analysis of a large set of tissue samples from T1D and non-diabetic organ donors showed that enterovirus protein was more frequent in tissues of T1D donors compared to control donors. In addition, the proportion of enterovirus RNA-positive samples was much lower than that of samples positive for the viral VP1 protein. Certain anti-viral response markers were also more strongly expressed in the infected islets of T1D donors compared to control donors, particularly those markers that reflect intercellular communication via interferon signaling, which may contribute to the immune-mediated pathogenesis of T1D.

TIIVISTELMÄ

Tyypin 1 diabetes on sairaus, johon ei ole parannuskeinoa eikä ennaltaehkäisevää hoitoa. Tyypin 1 diabeteksessa insuliinia tuottavat haiman beetasolut ovat vaurioituneet tai tuhoutuneet kroonisen autoimmuuniprosessin seurauksena, mikä johtaa elinikäisen insuliinihoidon tarpeeseen. Geneettinen alttius määrittelee taipumuksen sairastua, mutta tämän lisäksi tarvitaan ympäristötekijöiden vaikutus. Esimerkiksi lukuisat epidemiologiset tutkimukset ovat liittäneet enterovirusinfektiot tyypin 1 diabetekseen. Tämän tutkimuksen tavoitteena oli kehittää ja vertailla menetelmiä, joilla pystytään tunnistamaan enterovirus solu- ja kudosnäytteistä. Lisäksi tavoitteena oli soveltaa näitä menetelmiä diabeetikoiden ja esidiabeetikoiden kudosnäytteiden analysointiin enterovirusten osuuden selvittämiseksi tyypin 1 diabeteksen tautiprosessissa.

Kaupallista *in situ* hybridisaatio-teknologiaa käytettiin enteroviruksen genomin paikallistamiseen formaliinilla fiksatuista ja parafiiniin valetuista (FFPE) kudosleikkeistä. Enterovirukselle spesifisiä ja viruksen genomin tunnistavia koettimia suunniteltiin ja niiden toimivuus testattiin eri enteroviruksilla infektoiduilla soluviljelynäytteillä ja infektoitujen hiirten kudosnäytteillä. Menetelmä pystyi toteamaan viruksen näistä näytteistä ja tunnistuksen tarkkuutta voisiin säädellä koettimien sekvenssiä muuntelemalla siten, että menetelmä tunnisti joko laajakirjoisesti monia eri enteroviruksia tai vain yhtä enteroviruksen alatyyppiä.

Eri enteroviruksen tunnistusmenetelmien keskinäinen vertailu viruksella infektoitujen humaanisolujen laimennossarjassa (jää- ja FFPE –näytteet) osoitti, että jäänäytteissä herkimmät menetelmät olivat RT-qPCR tunnistaen enteroviruksen laimeimmastakin näytteestä (10^{-8}) ja proteomiikka (10^{-7}). Immunohistokemia kaupallisella enterovirus VP1-vasta-aineella tunnisti enteroviruksen FFPE-näytteistä 10^{-6} laimennoksesta ja ISH puolestaan 10^{-4} laimennoksesta.

RT-qPCR:ää ja immunohistokemiaa käytettiin Network for Pancreatic Organ Donors with Diabetes (nPOD) -projektin kudoskokoelman analysointiin seuraavista elinluovuttajaryhmistä: tyypin 1 diabeetikot (T1D), autovasta-ainepositiiviset

esidiabeetikot (AAb+) ja elinluovuttajat, joilla ei ollut diabetesta (verrokit). Parafiinileikkeitä haimasta, pernasta, ohutsuolesta ja haiman imusolmukkeista värjättiin kaupallisella enterovirus VP1-vasta-aineella. Värjäykset osoittivat, että enteroviruksen proteiinia löytyy diabeetikoiden kudoksista useammin kuin verrokkien; haimassa 70 % vs. 33 % (vain insuliinipositiiviset elinluovuttajat laskettu mukaan), ohutsuolessa 40 % vs. 14 % ja pernassa 40 % vs. 26 %. Haiman imusolmukenäytteitä analysoitiin vain 10 elinluovuttajalta (9 T1D ja 1 AAb+) ja näistä 56 % ja 100 % oli enterovirus-positiivisia. Lisäksi samoista kudoksista analysoitiin jäänäytteitä RT-qPCR:llä enteroviruksen genomin tunnistamiseksi. Enteroviruksen RNA:ta löydettiin kaikista muista kudoksista paitsi ohutsuolesta, mutta selvästi harvemmin kuin enteroviruksen proteiinia. Kun enterovirus-positiivisia haimanäytteitä värjättiin vasta-aineilla, jotka tunnistavat virusinfektiovasteeseen liittyviä soluproteiineja isäntäsoluissa (HLA I, MxA ja PKR), huomattiin, että diabeetikoiden enterovirus-positiiviset saarekkeet ilmensivät voimakkaasti HLA I:stä ja MxA:ta. Verrokeiden enterovirus-positiivisissa saarekkeissa näiden markkereiden ilmentyminen oli normaalia. Tämän perusteella voidaan spekuloida, että virusinfektion aikaansaamat sytokiinivasteet poikkeavat eri ryhmien välillä.

Yhteenvetona, työssä kehitettiin enteroviruksen RNA:ta solu- ja kudosnäytteistä tunnistava *in situ* hybridisaatiomenetelmä. Menetelmän kykyä tunnistaa eri enterovirustyyppejä pystyttiin säätelemään koettimien sekvenssiä muuntelemalla. Menetelmän herkkyys ei kuitenkaan ollut riittävä viruksen tunnistamiseksi humaanikudosnäytteestä, jossa viruksen määrä on alhainen. Diabeetikoiden ja verrokkien kudosnäytteitä analysoitaessa huomattiin, että enterovirukset ovat yleisempiä diabeetikoiden kudoksissa kuin verrokkien. Lisäksi, virus-RNA-positiivisten näytteiden lukumäärä oli selvästi pienempi kuin virusproteiinille positiivisten määrä. Lisäksi erityisesti interferonivälitteiseen solujenväliseen vuorovaikutukseen liittyvät virusvastetta ilmaisevat merkkiaineet olivat infektoituneissa saarekkeissa diabeetikoilla selvästi enemmän koholla kuin verrokeilla, mikä saattaa osaltaan vaikuttaa taudin immuunivälitteiseen syntyyn.

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original publications referred to in the text by Roman numerals I-III:

I. Laiho JE, Oikarinen S, Oikarinen M, Larsson P, Stone V, Flodström-Tullberg M, Isola J, Hyöty H. (2015) Application of bioinformatics in probe design enables detection of enteroviruses on different taxonomic levels by advanced in situ hybridization technology. J Clin Virol 69:165-171

II. Laiho JE*, Oikarinen M*, Richardson SJ, Frisk G, Nyalwidhe J, Burch TC, Morris MA, Oikarinen S, Pugliese A, Dotta F, Campbell-Thompson M, Nadler J, Morgan NG, Hyöty H, JDRF nPOD-Virus Group. (2016) Relative sensitivity of immunohistochemistry, multiple reaction monitoring mass spectrometry, *in situ* hybridization and PCR to detect Coxsackievirus B1 in A549 cells. J Clin Virol 77:21-28

III. Oikarinen M*, Laiho JE*, Oikarinen S, Richardson SJ, Pugliese A, Campbell-Thompson M, Morgan NG, Tauriainen S, Hyöty H. (2018) Detection of enterovirus protein and RNA in multiple tissues from nPOD organ donors with type 1 diabetes. bioRxiv 459347; doi: https://doi.org/10.1101/459347

* shared first authorship

ABBREVIATIONS

A549	carcinomic human alveolar basal epithelial (cells)
Ad	adenovirus
APC	antigen-presenting cell
ATCC	American Type Culture Collection
CAR	coxsackie-adenovirus receptor
CMA	cell microarray
CNS	central nervous system
CTL	CD8+ cytotoxic T lymphocyte
CV-A	coxsackievirus A
CV-B	coxsackievirus B
CXCL10	C-X-C motif chemokine ligand 10
dsRNA	double-stranded RNA
DAF	decay-accelerating factor
DiViD	The Diabetes Virus Detection study
E	echovirus
ELISA	enzyme-linked immunosorbent assay
EV	enterovirus
FFPE	formalin-fixed paraffin-embedded
GADA	glutamic acid decarboxylase antibody
GMK	green monkey kidney cells

GWAS	genome-wide association study
HFMD	hand, foot and mouth disease
HeLa	carcinomic human cervix epithelial cells
HIER	heat-induced epitope -retrieval
HLA	human leukocyte antigen
IA-2A	islet antigen 2 antibody
IAA	insulin autoantibody
ICA	islet cell antibody
ICAM-1	intracellular adhesion molecule 1
IF	immunofluorescence
IFN	interferon
IgSF	immunoglobulin superfamily
IHC	immunohistochemistry
IRES	internal ribosomal entry site
IRF1	interferon regulatory factor 1
ISG	interferon-stimulated gene
ISH	*in situ* hybridization
JDRF	Juvenile Diabetes Research Foundation
LC	liquid chromatography
MDA5	melanoma differentiation-associated protein 5
MHC	major histocompatibility complex
MRM	multiple reaction monitoring
MS	mass spectrometry
MxA	human myxovirus resistance protein 1

NGS	next generation sequencing
NOD	non-obese diabetic (mouse)
nPOD	network for Pancreatic Organ Donors with Diabetes
OAS	2′5′-oligoadenylate synthetase
OCT	optimal cutting temperature
O/N	overnight
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PLN	pancreatic lymph node
PKR	protein kinase R
PRR	pathogen recognition receptor
PV	poliovirus
PVR	poliovirus receptor
RIG-I	retinoic acid-inducible gene-I
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
RT-qPCR	reverse transcriptase quantitative PCR
RD	human rhabdomyosarcoma muscle cells
SNP	single nucleotide polymorphism
ssRNA	single-stranded RNA
STAT 1	signal transducer and activator of transcription 1
T1D	type 1 diabetes
TMA	tissue microarray
TNF	tumor necrosis factor

TYK2	tyrosine kinase 2
UTR	untranslated region
Vero	green monkey kidney cells
VP	viral protein
VPg	genome-linked viral protein
ZnT8	zinc transporter-8

1 INTRODUCTION

T1D is an important and complex disease, affecting millions of people worldwide but primarily in Western countries. It mainly affects children or young adults. The financial burden is massive, as a life-long insulin treatment, surveillance, and follow-ups are obligatory. The etiology of the disease is complex; it most likely has multiple triggers and, thus, varying pathways that ultimately lead to the same end point, the loss of insulin production. The insulin-producing beta cells of the pancreatic islets (Islets of Langerhans) are affected or destroyed during development of T1D. This leads to loss of insulin, which is needed to maintain the glucose homeostasis. Affected individuals face severe complications or even death if treatment is insufficient or comes too late. Since there is no cure or prevention for the disease, it is imperative to identify not only the cause(s) and trigger(s) of the disease, but also the underlying mechanism that ultimately results in the destruction of the insulin-producing cells.

This study focuses on one of the most important potential triggers of T1D, enteroviruses, and development of techniques that can be used to detect these viruses with high sensitivity and specificity. More importantly, the presence of enteroviruses in the tissue samples between T1D patients, prediabetic individuals and non-diabetic controls was compared. In addition, selected markers of anti-viral immune response were assessed in enterovirus positive pancreas to identify possible differences in host's antiviral response between T1D patients and control subjects.

2 REVIEW OF LITERATURE

2.1 Enteroviruses

2.1.1 Classification, structure and replication cycle

The genus Enterovirus belongs to the family *Picornaviridae*, one of the largest virus families. Picornaviruses are grouped into 40 genera, and the genus Enterovirus comprises 15 species: *Enterovirus* A-L and *Rhinovirus* A-C. Seven of these 15 species, namely *Enterovirus* A-D and *Rhinovirus* A-C, are associated with human diseases (Fig. 1). Different species comprise different enterovirus types. Enterovirus types are classified by sequencing a specific genomic region encoding the structural VP1 protein. Species A-D alone comprise altogether 116 different virus types that infect humans. The number of different enterovirus types has been constantly increasing over the years due to the sequencing of new clinical isolates and identification of new enterovirus types from ongoing studies. (1,2)

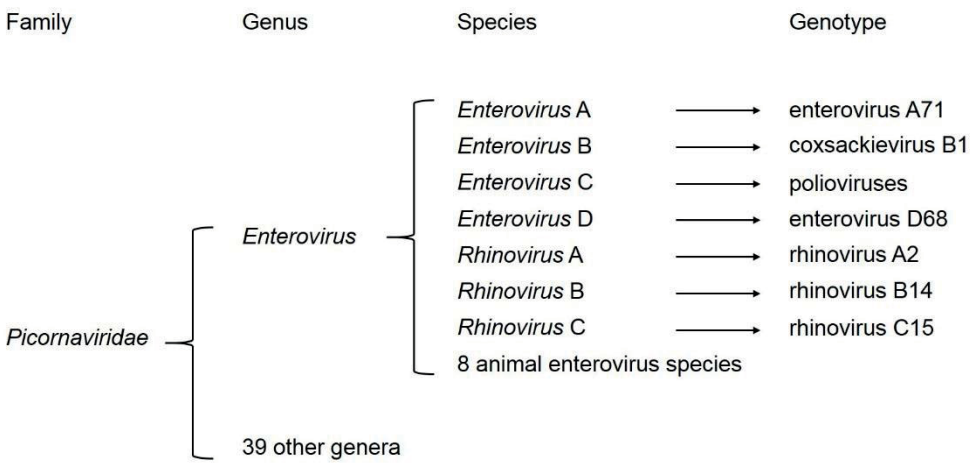

Figure 1. Classification of the family *Picornaviridae* and the genus *Enterovirus*. In the genus *Enterovirus* the seven species that infect humans are depicted. Representative types for each of the seven enterovirus species are shown.

The enterovirus virion, like other picornaviruses, is spherical and approximately 30 nm in diameter. The capsid surrounding the naked RNA genome has an icosahedral symmetry of 60 protomers. Each protomer comprises four capsid proteins called VP1, VP2, VP3 and VP4. VP1-VP3 are located on the surface of the capsid and VP4 on the internal side of the capsid (3) (Fig. 2 A). The major capsid proteins VP1-VP3 have a common topology, forming an eight-stranded beta barrel structure that is joint together by loops in variable lengths. The loop regions are also the sites where the main structural differences are found. Connecting loops project onto the surface of the virion where they make up the antigenic sites and give rise to the immunological diversity of virus types in *Enterovirus* genus (4,5).

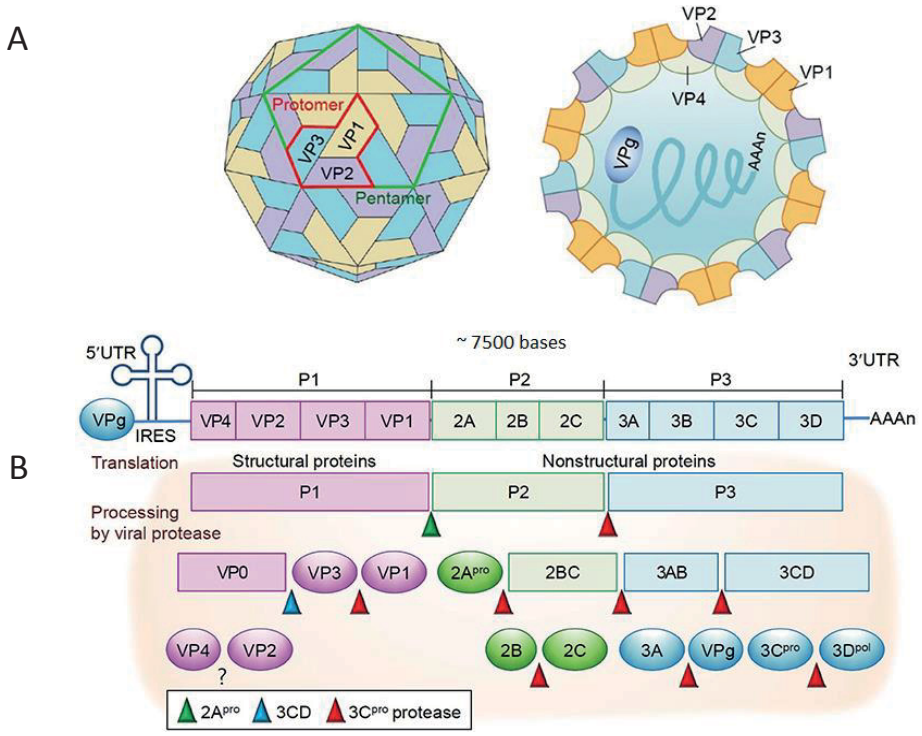

Figure 2. A. Enterovirus structure. Enterovirus is a non-enveloped particle with icosahedral symmetry, constituted of 60 copies of four structural proteins VP1-VP4. These VPs assemble into protomers (marked in red), five protomers form a pentamer (marked in green) and 12 pentamers plus the viral genome that is packaged inside the capsid form a virion. B. The RNA genome of enterovirus is approximately 7.5 kb in length with 5' / 3' UTRs and VPg / polyA in 5' and 3' ends, respectively. During viral replication, the RNA genome is translated into a polyprotein that is sequentially cleaved by viral proteases 2A, 3CD and 3C, to form mature structural and non-structural proteins. (Adapted from Yi et al 2017 (6))

Enteroviruses have a positive-sense, single-stranded RNA (+ssRNA) genome, which is approximately 7.5 kb in length. The genome has a single open reading frame (ORF) flanked by 5´and 3´untranslated regions (UTRs) and a small protein (VPg) covalently attached to the 5' terminus (Fig. 2B). The VPg is unique for picornaviruses and important in the RNA replication process (7). The coding region of enteroviruses is divided into three sub-regions, named P1, P2 and P3, where P1 encodes the structural proteins that form the outer capsid of the viral particle. Conversely, P2 and P3 encode seven non-structural proteins that are responsible for the enzymatic processes, such as the proteolytic cleavage that releases the structural proteins and the RNA-dependent RNA polymerase (Fig. 2) (6).

The primary replication of enteroviruses occurs in cells of mucosal tissue of gastrointestinal and respiratory tracts. Replication cycle begins when the virus recognizes and binds a host receptor, which mediates the endocytosis of the virus. Viral RNA is released into the cytoplasm after entrance and is directly translated to form a polyprotein from the viral genome´s single open reading frame (ORF). Polyprotein is cleaved by virus-encoded proteases to produce the final virus proteins. RNA synthesis occurs in special replication organelles or vesicles, where the (+)ssRNA genome is copied through a negative strand RNA intermediate, which is then used as a template for new viral genomes. Double-stranded (dsRNA) intermediate molecules are also formed at this stage. The final steps of replication cycle include packaging the newly formed RNA inside the viral capsid to form new infectious particles and the release of these particles outside the host cell. Fig. 3 details the different steps of the replication cycle. Viral proteases $2A^{pro}$ and $3C^{pro}$ are the main proteases responsible for the cleavages of the polyprotein into structural and nonstructural proteins that eventually form the capsid structure. The cleavage processes of $2A^{pro}$ and $3C^{pro}$ are not limited to the viral protein processing only, but they also cleave several other cellular factors and protein targets to support virus reproduction and also to optimize the environment for viral proliferation. (8,9)

Different enterovirus types recognize different cellular receptor(s). The capsid proteins are responsible for the binding to a cell receptor, which then initiates the infection. Many enteroviruses use similar receptor molecules that belong to the immunoglobulin superfamily (IgSF). IgSF-like receptors include for example poliovirus receptor (PVR or CD155) for polioviruses (PVs), coxsackie-adenovirus receptor (CAR) for coxsackie B viruses (CV-Bs) and intercellular adhesion molecule-1 (ICAM-1) that is used by coxsackievirus A21 (CV-A21) and many rhinoviruses. The extracellular regions of IgSF receptors have two to five N-terminal immunoglobulin-

like domains, of which the outermost domain, D1, is involved in the binding with the conserved amino acid residues of a special region called “the canyon” of the virus capsid. Some enteroviruses may also use non-IgSF cell surface receptors. These receptors include the decay-accelerating factor (DAF or CD55) and a number of integrins, which are used by certain echoviruses and CVBs, and heparan sulfate that is used by many hand foot and mouth disease (HFMD) viruses, such as CV-A16 and EV-A71 (10).

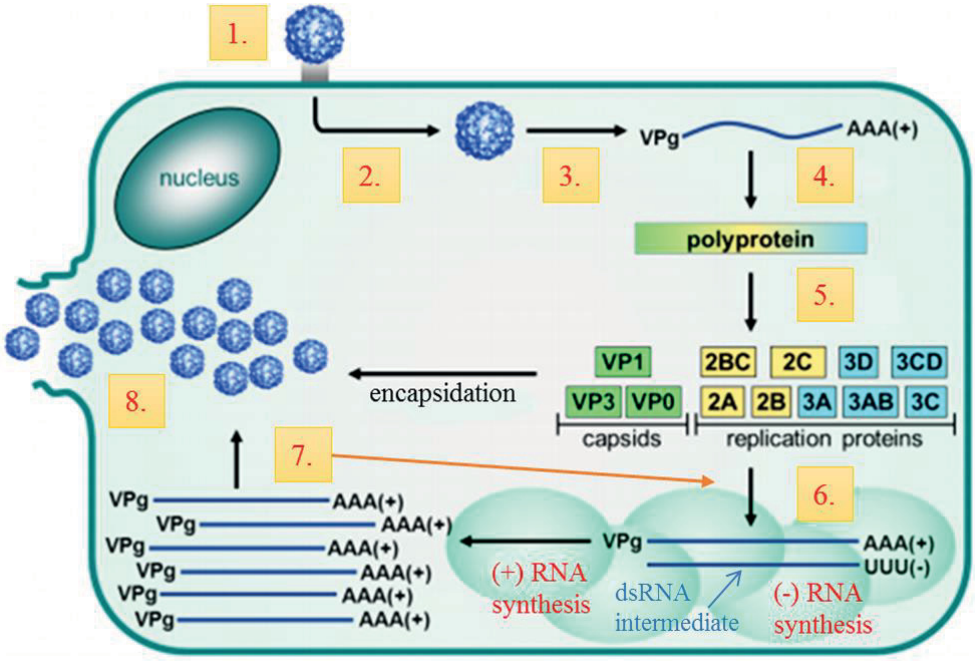

Figure 3. Enterovirus replication cycle: 1. Binding of virus to receptor e.g.. CAR, DAF. 2. Internalization of the virus. 3. Release of RNA genome from the virion. 4. Translation into polyprotein 5. Proteolytic processing by viral proteases into viral proteins. 6. Replication of the RNA genome via a dsRNA intermediate mediated by the nonstructural proteins in replication organelles or vesicles. 7. Newly synthesized positive-stranded RNA molecules either enter another round of replication (orange arrow) or are packaged into the viral capsid proteins to form new infectious particles. 8. Release of new infectious particles by cell lysis or non-lytic mechanisms. Modified from van der linden et al 2015 Viruses. (8)

2.1.2 Enterovirus diseases

Enteroviruses are very common around the world, usually causing mild asymptomatic infections. Transmission happens via fecal-oral or respiratory routes. Enterovirus circulation follows a seasonal pattern in temperate climates, where enteroviruses are found during the summer and early months of the fall. In tropical areas, however, enteroviruses can be found constantly throughout the year, or they are associated with the rainy season (9,11).

Regardless of the fact that most enteroviral infections are mild, infection can also lead to acute illnesses, such as herpangina and HFMD (12). Acute severe diseases result from virus spreading from the primary replication site to other target organs. These severe diseases include acute flaccid paralysis, pericarditis, myocarditis, meningitis and encephalitis (9,13,14). Young age and male gender increase the risk of severe diseases (9). Enteroviruses have also been linked to certain chronic diseases, such as chronic dilated cardiomyopathy (15) and T1D (16-19). The role of enteroviruses in T1D is discussed further in Chapter 2.6.

2.2 Diagnosis of enterovirus infections

Reliable diagnosis of enterovirus infection requires laboratory methods to detect the virus or virus-induced immune responses. Several assays are available. The sensitivity to diagnose enterovirus infections depends on the selection of the assay and collection of optimal types of samples. For example, acute infection can be diagnosed by detecting the virus directly from clinical samples while serological assays are used to detect recent infection and immunity. During acute infection, virus titers are usually high in stools and nasopharyngeal mucosa but the virus can also be detected in cerebrospinal fluid during central nervous system infection and in the skin blisters during HFMD. Virus can also be detected in tissue samples, e.g. in brain tissue during acute encephalitis and in myocardium during acute myocarditis. Thus, enteroviruses can be detected from nasal and throat swabs, cerebrospinal fluid, stool, tissues and blood samples. Different diagnostic methods are described below.

2.2.1 Virus Isolation

Enteroviruses can be isolated from different sample types (i.e. stool, cerebrospinal fluid, nasopharyngeal aspiration, tissue, blood). Isolation requires sterile cell culture

conditions and cell lines that support the virus replication. Samples are incubated in cells and observed regularly to detect possible virus replication either by visual inspection (monitoring cell death or formation of cytopathic effect) or by using enterovirus-specific antibodies and immunofluorescence microscopy. Reliable diagnosis requires confirmation of the presence of enterovirus by preventing virus replication using enterovirus specific hyperimmune sera, detecting enteroviral proteins using immunostaining or detecting viral nucleic acids using RT-PCR. The advantage of virus isolation is that the isolated virus becomes available for further studies. The main limitations include the facts that the assay is labor-intensive, the virus needs to retain its infectivity in the original sample and many enteroviruses do not replicate efficiently in commonly used cell lines. (20) Due to these limitations virus isolation has largely been replaced by RT-PCR based assays and is currently only rarely used in routine diagnostic laboratories.

2.2.2 RT-PCR

Currently, the most common method used to detect enteroviruses in clinical virus laboratories is reverse transcription PCR (RT-PCR) (21,22). In RT-PCR, viral RNA is first transcribed into complementary DNA (cDNA), which in turn is subsequently used as a template for the PCR reaction (23). This principle is the basis of amplification of virus genome using virus specific primers, providing high sensitivity and specificity for the assay. Various modifications of RT-PCR exist. Traditional assay includes 30-40 cycles of PCR amplification followed by the detection of enterovirus-specific PCR products by inspecting the bands of right molecular size in gel electrophoresis or by detecting enterovirus specific sequences using oligonucleotide probes or sequencing. In nested and semi-nested PCR, the amplicon of the initial PCR is amplified using another primer pair. During the past decades, real-time PCR technologies have revolutionized the detection of enteroviruses. These technologies are based on labelled probes (such as TaqMan), which emit fluorescing signal in correlation to the amount of PCR amplicons in the PCR reaction. Advantage of this method compared to gel electrophoresis is that the probe detects the amplicons according to sequence, which confirms that the detection is specific for the target of interest (24). These technologies allow the quantification of the viral RNA using fluorescent labeling and continuous monitoring of amplification as PCR reaction progresses. This makes it possible to quantify the number of copies of viral genome that are present in the sample. Accurate quantification requires standards with known amount of viral genome. Due to the quantitative nature, this kind of assay is called quantitative PCR (qPCR). The primers that are used to reverse

transcribe and amplify enterovirus RNA, influence the sensitivity of RT-PCR. Enterovirus 5'UTR is highly conserved between different enterovirus types, and primers targeting this region enable a sensitive, specific and effective detection of a wide range of different enterovirus types/species. For this reason, 5'UTR RT-qPCR is also recommended as a primary assay for enterovirus detection (25). Amplification of viral genome offers also possibilities for molecular typing of detected enteroviruses by sequencing the PCR products. Depending of the sequenced genome region this method can be used to classify enteroviruses in different genetic clusters ranging from two clusters (conserved 5'UTR) to the individual types (VP1 coding region corresponding to traditional serotype of the virus) (26-28).

2.2.3 Immunohistochemistry

Antibody-based methods can be used for the detection of enteroviruses in cell and tissue samples, for example using immunohistochemistry (IHC) (29-31). One major advantage of IHC is the ability to visualize and localize the virus antigen directly within cells and tissues. IHC is based on specific binding of specific antibodies to the target antigen and visualization of this binding by labelling the bound antibody. Enzyme- and fluorophore-mediated chromogenic and fluorescent detections, respectively, are the most popular methods. In chromogenic IHC, an enzyme label (such as alkaline phosphatase (AP) and horseradish peroxidase (HRP)) is allowed to react with its substrate (such as 3,3'-diaminobenzidine (DAB)) to yield an intensely stained precipitate. In immunofluorescence (IF) detection, organic fluorophores or fluorescent dyes are conjugated to the primary or secondary antibody (32).

The steps and reagents included in IHC depend on the sample type, target in question and the nature of target-specific antibody. For paraffin sections deparaffinization and subsequent rehydration is necessary. Most antigens and antibodies require an antigen retrieval method, which renatures the proteins to their pre-fixation conformation. Primary antibodies can be directly labeled with a dye (direct method), or a secondary antibody conjugated with the label can be used for the detection (indirect method). Sample staining can be performed manually or by using an automated system, and viewed with a brightfield or fluorescence microscope, depending on the staining methodology applied (chromogenic vs. fluorescing label) (32).

Staining of the samples is usually performed on microscope slides to which thin tissue and/or cell samples have been placed (works for both fixed samples and frozen tissue sections). Cell or tissue microarrays (CMAs or TMAs) are beneficial in

terms of time and reagents used as several samples can be co-stained. These microarrays can be made by drilling small cylindrical punches from donor paraffin blocks, using a syringe needle, and placing them to a recipient paraffin block. (33)

Currently, the commercially available mouse monoclonal antibody clone 5D8/1 from Dako (M7064, Agilent) is the most widely applied for the detection of enterovirus proteins by IHC. This antibody recognizes an epitope from the N-terminal region of the capsid VP1 protein and can be used for formalin-fixed, paraffin-embedded (FFPE) samples (34,35). Clone 5D8/1 recognizes a wide range of enteroviruses from species B, such as CV-Bs and echoviruses 3, 6, 9, 11 and 30 (36). However, some echoviruses, such as echovirus 2, 8 and 19, and EV68-71 (37) are not recognized. Potential cross-reactivity of clone 5D8/1 with host proteins has recently been discussed (38,39). However, it has been shown that cross-reactivity is not an issue when the antibody is used in optimized conditions (40). Other commercially available enterovirus antibodies are also available, such as ready-to-use enterovirus screening-set (Ref. 3465, Light Diagnostics, EMD Millipore Corporation), which contains six different monoclonal antibodies against different enterovirus groups. Additional broad-recognizing enterovirus antibodies would be beneficial to verify the enterovirus findings obtained with clone 5D8/1. However, the availability of such antibodies is limited, although attempts have been made for their production (39,41,42).

2.2.4 *In situ* hybridization

In situ hybridization (ISH) can be used to detect and localize viral RNA in infected tissues. This technique is mainly used for FFPE samples. ISH is based on the use of labelled probes that bind specifically to the target RNA or DNA during the hybridization step (43). ISH is a demanding method, which includes several steps and is more sensitive to errors compared to IHC, in which antibodies are used. Traditional ISH techniques include radioisotope-labeled probes, but nowadays non-isotopic ISH applications are mostly used. However, they lack the sensitivity and specificity required to measure low-abundance biomarkers reliably (44). Novel commercially available ISH techniques have recently arrived on the market, such as QuantiGene® ViewRNA by Invitrogen (previously Affymetrix) and RNAscope by ACD Biosystems (45). The workflow in these novel ISH techniques includes four main steps: sample preparation, target hybridization, signal amplification and detection (Fig. 4). These techniques promise even single molecule detection of multiple targets in single cells in optimal conditions, due to a fluorescent ISH and a sequential branched DNA amplification technique. The techniques also enable a customized

probe design for the target in question and parallel detection of multiple targets by using a unique label (color) for each target. Different kits are available for cell and tissue preparations, and currently one or two targets can be stained and visualized simultaneously in tissue samples, whereas cell preparations offer simultaneous visualization of up to four different RNA targets (43,45).

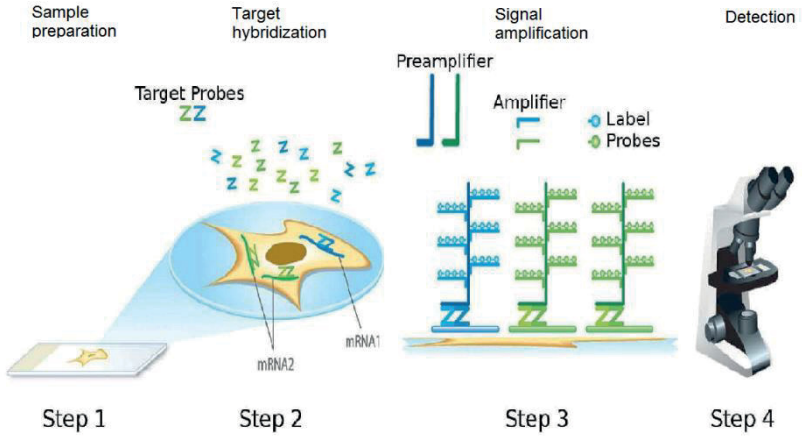

Figure 4. Schematic drawing on the workflow of novel ISH tecniques that base on branched DNA technology and subsequent amplification steps. In this example, two different targets are detected. In step 1 cells or tissues are fixed and permeabilized to allow the designed probe access its target. In the drawing, the blue and green colors represent different target probes. In step 2, target RNA-specific oligonucleotide probes (Z) are hybridized in pairs (ZZ) to multiple RNA targets. Only ZZ pairs will produce a signal in the subsequent amplifications steps. In step 3, signal is amplified with specific amplifier molecules, each specific for their target probe. Each label probe is conjugated to a different fluorophore or enzyme. In step 4, signals can be detected using a brightfield or fluorescence microscope, depending on the assay. (Modified from Wang et al. (2012) J. Molec. Diag. (45))

2.2.5 Other enterovirus detection methods

Next generation sequencing (NGS) methods allow an unbiased detection of any virus, including enteroviruses. NGS techniques produce a large amount of sequence data, increasing the likelihood of finding a small amount of virus sequence from the sample. The challenges of NGS techniques include the capacity of managing the computational data and cost of processing the samples (46), though the costs have come down substantially during the past few years. In addition, the sensitivity of NGS in virus detection is usually lower compared to specific PCR.

Proteomics has not been widely used for the detection of enteroviruses. In proteomics, proteins are studied on a large scale in biological systems. Studying proteins that are produced by the cell or that are present in a cell is useful, since not all mRNA is translated into protein; thus, simply studying the genomic contents would not tell the whole story about proteins present in the cell. Proteomics can also overcome and identify post-translational modifications of proteins that are critical to the protein's function. Therefore, proteomics confirms the presence and nature of the protein, and provide a direct measure of the quantity of the protein present. Numerous sample types, such as tissue, cell lines, enriched cell organelles and laser-captured micro-dissected cell populations, can be analyzed. Several proteomics approaches already exist; recently, these techniques have evolved enormously, leading to high sensitivity and specificity when digesting specific peptides from the samples. Regardless of these advantages, proteomics technologies are new and expensive and not yet routinely applied in enterovirus diagnostics. One of the most sensitive technologies is a liquid chromatography multiple reaction monitoring mass spectrometry (LC/MRM/MS/MS) on a triple quadruple (QqQ) mass spectrometer. It provides a rapid, sensitive, and specific identification and quantitation of targeted compounds in complex samples. The technological basis of this system in a QqQ mass spectrometer provides the capability to identify an ion of interest (Q1 m/z), followed by the ability to segregate and fragment that precursor ion in the collision-induced chamber (in Q2). It also enables the identification of the ion of interest from the fragmented precursor ion (Q3 m/z), and only detects ions with this exact transition (47,48).

2.2.6 Detection of enterovirus-specific serum antibodies

Enterovirus infection can be diagnosed by detecting enterovirus-specific antibodies from serum or other body fluids. Detection of enterovirus-specific IgM can be used to diagnose acute or recent infection from a single serum sample. Increases in IgG titers can be used to diagnose infection using paired sera (acute and convalescence serum). The most widely used method is enzyme-linked immunosorbent assay (ELISA). It is a fairly simple and low cost technique that can be used to detect antibodies against enteroviruses in different immunoglobulin classes. The antibodies that bind to certain enterovirus antigen in ELISA are not specific for that enterovirus type but represent heterotypic antibodies that cross-react widely between different enteroviruses (49).

Enterovirus infection induces antibodies that neutralize the infectivity of the virus. Acute infection can be diagnosed by showing an increase in neutralizing antibody titers between paired serum samples. These antibodies stay elevated for a long time after the infection and provide protection against reinfection. They are type-specific and can be used to identify the type of enterovirus causing the infection. Therefore, hyperimmune animal sera containing high titers of neutralizing antibodies against specific enteroviruses have been used to identify the type of enteroviruses detected by virus isolation in cell cultures. Presence of neutralizing antibodies in a single sample reflects the infection history of an individual, although in some cases also transient antibody responses can occur, especially if the infection has been associated with a low virus titer (50). Serum is considered seropositive if it can inhibit a virus-induced cytopathic effect or the formation of plaques in cultured cells (51). Thus, the assay requires sterile cell culture work and is expensive and labor-intensive. Therefore, it is not used in routine diagnostics.

2.3 Immune response against enteroviruses

Mammalian immune system is remarkably complex, enabling efficient recognition of invading pathogens and impaired host proteins that threaten the host homeostasis. Generally, the immune system is divided into two systems based on their functions in host defense: innate and adaptive immunity. While innate immunity provides robust, immediate and non-specific immune responses through i.e. complement activation and pathogen engulfment (52), adaptive immunity is organized around T and B lymphocytes that orchestrate diverse repertoire of antigen-specific recognition receptors and long-lived immunological memory against reinfection (52). Despite being timely, temporally and mechanistically separate systems, innate and adaptive immune system have complex interactions that regulate their function.

When an enterovirus encounters the body, it first infects cells in intestinal mucosa or upper respiratory tract and consequently spreads to local lymph nodes underneath the mucosa. The virus may also spread to secondary organs, such as the heart, pancreas and central nervous system (CNS), although the mechanism is not fully established. The first rapid defense against enterovirus infection is triggered when pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs) and retinoic acid-inducible gene (RIG-I)-like receptors, recognize enterovirus-specific pathogen-associated molecular patterns (PAMPs) (53). Enterovirus-specific PAMPs

include ssRNA and dsRNA. Enterovirus that releases its ssRNA genome in the cells becomes recognized by TLR7 and TLR8 molecules. dsRNA is formed as a replication intermediate when the virus starts replicating and is recognized by intracellular vesicle-associated TLR3 or by the sensor melanoma differentiation-associated gene 5 (MDA5) (54). This leads to synthesis of cytokines, like type I interferons (IFN), in infected cells, with strong antiviral activity creating an antiviral state in the surrounding cells by the expression of proteins with antiviral activity (55). For example, the lack of MDA5 in a knockout mouse model leads to deficient type I IFN production and increased early mortality in CV-B infection (56). Overall, genes that interfere with virus replication prevent spread to neighboring cells and activate the adaptive immune response (57).

Innate immune responses also regulate adaptive immune responses. In fact, antigen-presenting cells (APCs) are also activated by PAMPs after which they present virus antigens to antigen-specific effector cells of the adaptive immune system. The adaptive B cell response produces and controls the production of antibodies against the infective virus during acute infection, and protects against reinfections by the same virus type (55,58). These antibodies inactivate enterovirus by neutralization and opsonization or commence the destruction of the infected cells by activating the complement (58). Antibody response seems to be a particularly important factor in the eradication of enterovirus infection and in the immunity against re-infections by the same serotype (presence of neutralizing antibodies correlate with protection) (59), while the role of T cell mediated immune responses is less well characterized. However, at least $CD4^+$ T cells recognize virus antigens that are bound to class II HLA molecules and can facilitate wide range of immune responses including the production of antibodies by B lymphocytes (Th2 type cells) and production of cytokines and chemokines (Th1 and Th17 type cells) that can mediate inflammation. CD8+ T cells, in turn, are activated by virus antigens presented by HLA class I molecules and can have direct cytotoxic effects on virus-infected cells (60).

Enteroviruses induce strong cell-mediated immune responses. However, their role in the eradication of the virus and in immune protection is less characterized compared to that of antibody responses. For example, memory T cells cross-react between different enterovirus types, generating anamnestic responses that speed up the eradication of the virus (55). Enteroviruses can cause a severe and/or chronic infection in immunocompromised and immunosuppressed individuals, or neonates who have an immature immune system (61). Humoral immunodeficiencies increase

the susceptibility to enteroviruses more than T cell deficiencies suggesting an important role of neutralizing antibodies in immune defense against enteroviruses (62). Such severe infections may lead to encephalitis or myocarditis and, in extreme cases, to death.

2.4 Pathogenesis of type 1 diabetes

Type 1 diabetes (T1D) is a life-long disease in which insulin-producing pancreatic beta cells are selectively impaired or destroyed. Historically T1D was referred to as juvenile diabetes due to the high incidence of the disease among children. This term is no longer recommended, since it is known that T1D can occur at any age, and about 50% of the cases are diagnosed at over 30 years of age (63). The incidence and prevalence of T1D varies substantially: Finland leads the statistics with the incidence of over 60 (>60 cases per 100 000 children each year). The prevalence of T1D is generally higher in countries with European ancestry; however, the disease incidence has wide variations even in neighboring areas in Europe and North-America (64). The incidence of T1D is very low (0.1 cases per 100 000 people each year) in countries like China, India and Venezuela (65). The incidence of T1D has been increasing during the past decades, although fluctuation or even plateau phases have also been observed (66). It is unclear, however, what the reason is for the ongoing increase, but changes in nutrition, virus infections, hygiene and gut microbiome have been given much attention (65,67)

At cellular level, the process leading to T1D is associated with an infiltration of the immune system cells into the islets of Langerhans, where insulin-producing cells are located. Genetic predisposition is a risk factor, but genes only partly explain the T1D incidence rates. This is supported by the fact that less than 10% of the children carrying the HLA risk genes will develop T1D (68), and studies performed on monozygotic twins have also shown less than 35% concordance rate for T1D (69,70), therefore indicating additional input of non-genetic factors. Consequently, other factors besides genetic factors trigger disease progression. It is common in many autoimmune diseases that both genes and biological factors make the individual susceptible for disease progress, and this occurs during the lifespan. Presently, it is well accepted that T1D is a disease that may have multiple origins, and environmental factors such as nutrition, vitamins, toxins or viral infections have been proposed to play a significant role in the pathogenesis of T1D. Specifically, enteroviruses are currently considered the major triggers of T1D development (see chapter 2.6).

2.4.1 Autoimmunity

In T1D, pancreatic beta cells are destroyed through a process characterized by an inflammation of the pancreatic islets and the appearance of autoantibodies that bind to beta cell autoantigens. Despite the fact that the exact mechanism leading to this destruction remains unclear, it is evident that normal immune system reacts against beta cell autoantigens. The disease progression is usually relatively slow and initiated long before the clinical T1D is diagnosed. The appearance of autoantibodies that target pancreatic islet antigens characterizes the initiation of the disease process. Autoantibodies against insulin (IAA), insulinoma-associated protein 2 (IA-2A), glutamic acid decarboxylase (GADA) or zinc transporter 8 (ZnT8A) can be found from peripheral blood during this prediabetic phase (71). All these antigens are localized in secretory granules of the beta cells (72), and all except insulin can also be found in some other cells and tissues. The time from seroconversion to autoantibody-positivity and subsequent development to T1D may take from a few weeks to several years. However, not all autoantibody-positive individuals will develop T1D. The risk for developing the disease generally increases with the number of autoantibodies detected, and the majority of children who are positive for two or more of these autoantibodies will develop T1D (73).

2.4.2 Genetics

The first T1D genetic susceptibility locus, the human leukocyte antigen (HLA) on chromosome 6p21 (Fig. 5), was found in the 1970s. Before the genome-wide association studies (GWAS) five other loci were established to be associated with T1D. With the development of high-throughput, single nucleotide polymorphism (SNP) genotyping arrays and GWAS systems, the number of T1D susceptibility genes has risen to almost 60 (74). Recent data have shown, for instance, that SNPs in genes that are involved in the immune response against viruses, such as MDA5 and tyrosine kinase 2 (TYK2), are risk factors for T1D (75-79). The HLA class II genes account for approximately half of the genetic risk for T1D (80) despite the high number of candidate genes. The polymorphisms in HLA class II genes encoding HLA-DQ and HLA-DR (Fig. 5) are linked to the highest T1D risk. Such haplotypes include HLA-DR3-DQ2 and HLA-DR4-DQ8, and the risk is the highest for the heterozygote formed by these two haplotypes. Some haplotypes, such as DR2-associated haplotypes, have in contrast been shown to protect from the disease (80). The HLA genes encode highly polymorphic proteins, which are essential to self- *versus* non-self-immune recognition. The HLA class II molecules are expressed in APCs, such as

dendritic cells and macrophages, which introduce the pathogen antigens to CD4+ T cells. This interaction promotes inflammation reaction via secreted cytokines. Conversely to HLA class II, the HLA class I molecules are expressed in virtually all nucleated cells, where they function by presenting intracellular antigens to CD8+ T cells. HLA class III genes encode proteins that are involved in the complement activation pathways, such as C2, factor B (Bf) and C4. Genes for the heat shock proteins and tumor necrosis factor (TNF) are also located in the HLA class III region. The physiological role of most of the HLA class III genes is yet to be determined, but some genes in this region are already linked to certain diseases, including T1D and Alzheimer's disease (80,81).

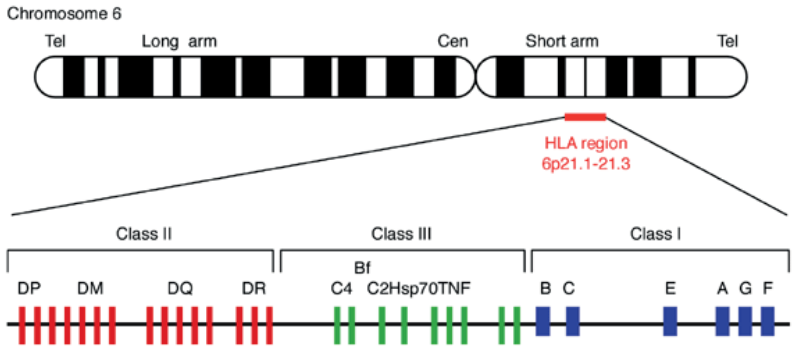

Figure 5. Gene map of the HLA region on human chromosome 6. HLA region, with class II, class III and class I genes, is located in the short arm of chromosome 6. HLA class I molecules control CD8+ cytotoxic T cell function and mediate immune responses against 'endogenous' antigens and virally infected targets, whereas HLA class II molecules are involved in the presentation of 'exogenous' antigens to CD4+ helper T cells. HLA class III region contains many genes encoding proteins that are unrelated to cell-mediated immunity, yet modulate or regulate immune responses by unknown manner. These include TNF, heat shock proteins and complement proteins (C2, Bf and C4). Adapted from Expert Reviews in Molecular Medicine C 2003 Cambridge University Press.

2.4.3 Pancreas pathology

The human pancreas is located in the retroperitoneal space of the abdominal cavity behind the stomach in close proximity to the duodenum and spleen (Fig. 6). The pancreas is a multifunctional organ consisting of acinar cells that produce digestive enzymes in the exocrine pancreas and glucose homeostasis hormone-producing cells in the endocrine pancreas, i.e., in the islets of Langerhans. The exocrine compartment comprises 98%, and the endocrine compartment comprises only approximately 2% of the whole pancreas.

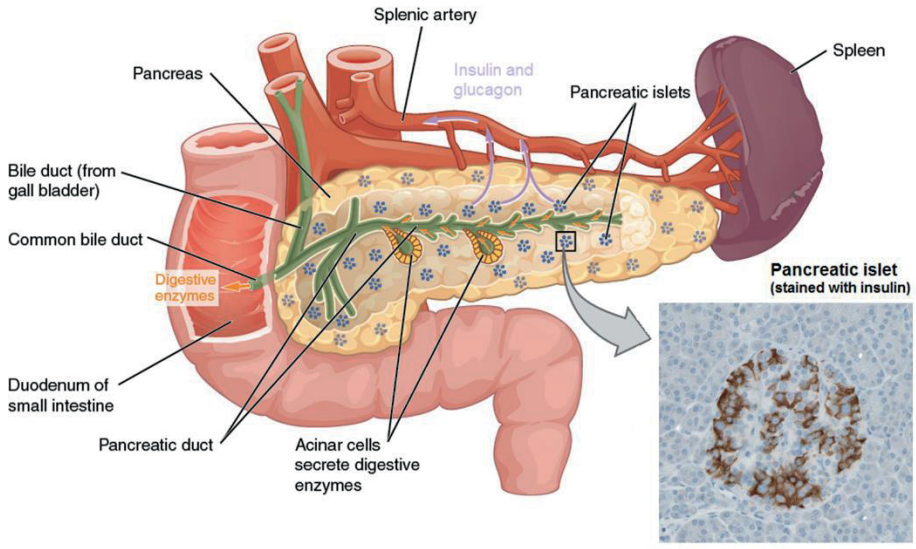

Figure 6. The human pancreas and its surroundings. Pancreas is located in close proximity to small intestine and spleen. Exocrine pancreas comprises acinar cells that produce and secrete digestive enzymes. The endocrine pancreas comprises Islets of Langerhans or pancreatic islets that are distributed throughout the pancreas. In the insert, a representative image of an islet immunostained for insulin. Adapted and modified from Anatomy and Physiology, an OpenStax resource at http://philschatz.com/anatomy-book/contents/m46685.html.

The endocrine cells, which are clustered into 'islets', comprise five different hormone-producing cell types: alpha cells that secrete glucagon, beta cells that secrete insulin, delta cells that secrete somatostatin, PP cells that secrete pancreatic polypeptide and epsilon cells that secrete ghrelin (Fig. 7) (82). Islets are distributed throughout the whole pancreas, contain approximately 1500 cells each, and are

150-300 µm in diameter (83). A vast vascular network of small capillaries runs through islets, which are also innervated by the symphathetic, parasymphatetic and sensory nervous system (84). The larger islets contain afferent arterioles, which branch from intralobular arteries in the exocrine tissue (85).

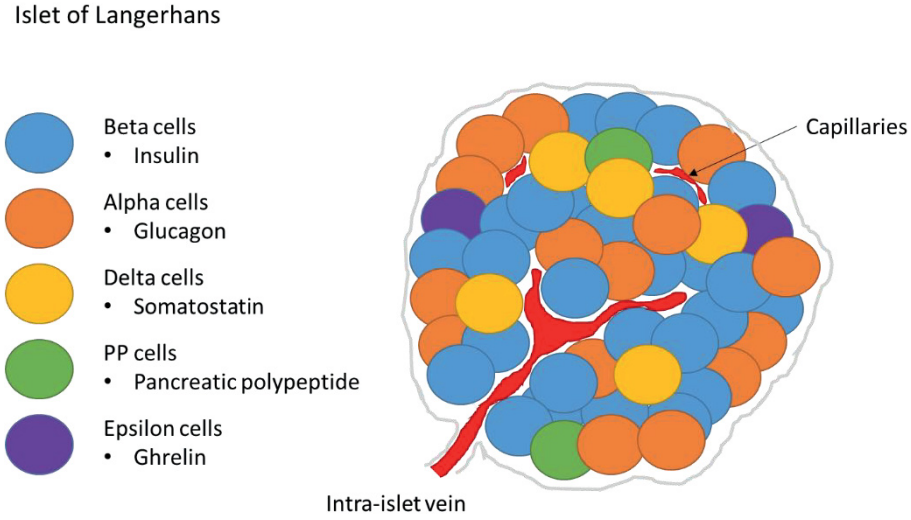

Figure 7. Schematic drawing of an Islet of Langerhans. The endocrine compartment of pancreas comprises only about 2% of the whole pancreas and is composed of Islets of Langerhans. In these islets, five different cell types produce five different hormones, insulin-producing beta cells being the most abundant. Veins and small capillaries are also abundantly present. Islets also contain afferent arterioles (not shown) which supply and regulate the blood flow (85).

T1D results from the specific destruction of pancreatic beta cells, which is, according to the current understanding, preceded by an inflammatory infiltrate targeting the islets. This phenomenon is known as “insulitis”, which consists predominantly of T cells dominated by CD8+ lymphocytes but also CD4+ lymphocytes, B lymphocytes and macrophages (86). Insulitis is defined as an event where at least three islets in a pancreatic section having 15 or more CD45+ lymphocytes infiltrate the islet (87). CD45 is a common leucocyte marker that recognizes both T and B cells. Insulitis lesion mainly affects islets with residual beta cells but it has occasionally also been observed in pseudoatrophic (insulin-negative) islets (88,89). In recent studies, different insulitic profiles have been identified in patients diagnosed with T1D at younger age and for patients diagnosed at adolescence or an older age. These

profiles are categorized according to the number of CD20+ lymphocytes in the infiltrate or, more specifically, according to the ratio of CD20+:CD4+ lymphocytes. A patient is categorized as CD20Hi if this ratio is >1.0 and as CD20Lo if the ratio is <1.0. Patients diagnosed before age seven always express the CD20Hi profile and have a more aggressive form of beta cell loss, whereas patients diagnosed at or above the age of 13 always display CD20Lo profile, with less aggressive loss of beta cells (90). The lymphocyte infiltration to islets is substantially rarer than the infiltration occurring in other autoimmune diseases, since only a small fraction of islets is affected by this phenomenon, and the number of infiltrating lymphocytes is quite small. Another early phenomenon of initiated autoimmune process is class I HLA (human leucocyte antigen or major histocompatibility complex, MHC) hyperexpression of the islets (see chapter 2.6.5).

2.5 Mechanism of virus-induced autoimmunity

The prevalence of many autoimmune diseases is increasing worldwide, but their etiology often remains to be determined. It is evident that environmental factors strongly influence the initiation and development of these diseases, although genetic predisposition plays an important role. Viral infections have been identified among the most potential autoimmunity triggers.

Molecular mimicry is one of the suggested mechanisms of autoimmunity. Viruses may contain peptides that are similar in their amino acid sequence or structure to self-antigens. It is hypothesized that when immune cells are activated in response to the virus, they may also recognize self-antigens and, thus, initiate an immune response that finally leads to destruction of these self-antigens, leading to autoimmunity (91). On the other hand, epitope spreading can occur due to collateral damage and active release of self-antigens caused by the immune response against the virus. APCs process and present viral and self-antigens to T cells, which in turn initiates an autoimmune process. Bystander activation of autoreactive T cells has also been proposed as a reason for inflammation (92). During bystander activation, HLA I molecules present viral antigens to virus-specific T cells that migrate to areas of infection. HLA I is a heterodimer, consisting of type I integral membrane glycoprotein heavy chain (HC) complex in association with soluble β_2-microglobulin (β_2M) (Fig. 8 A). All nucleated cells express HLA I molecules on the cell surface, where they present peptide fragments derived from the degradation of intracellular or viral proteins. Peptides are loaded in the peptide-binding groove of the HLA I – β_2M

complex on the cell surface, where they are constantly monitored by CD8+ cytotoxic T lymphocytes (CTLs). These CTLs mount an immune response upon the recognition of the peptide by T cell receptor (TCR), which kills the infected cells (93) (Fig. 8 B). Therefore, when CTLs and macrophages release cytokines at the inflammation site, the process may also lead to bystander killing of the uninfected neighboring cells. Persistent virus infections, where viral proteins are expressed at very low levels without cell lysis, may also lead to immune-mediated injury, since the viral antigen is constantly present sustaining the immune response. Persistent viral infections have been linked to at least myocarditis, multiple sclerosis and T1D (53).

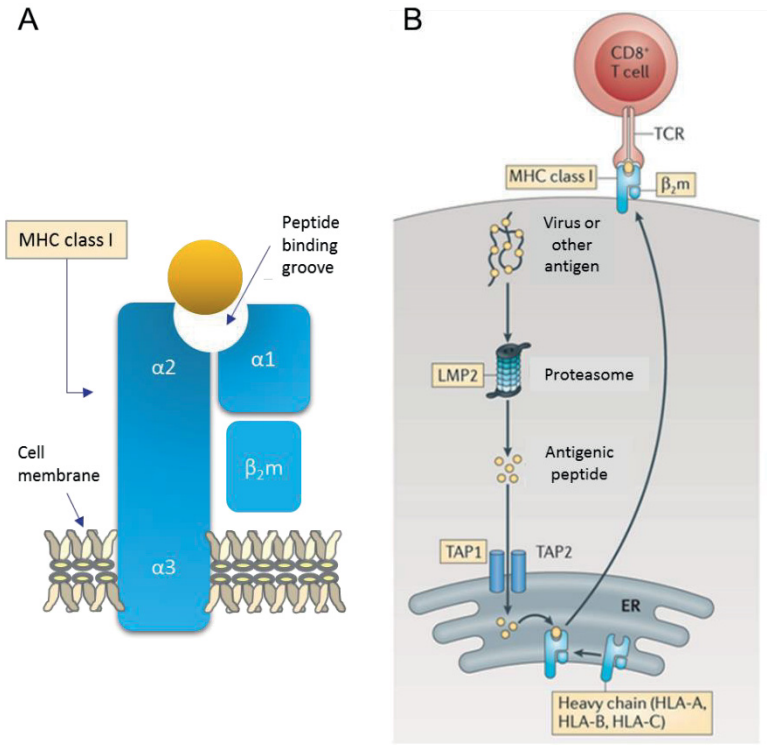

Figure 8. Structure and location of the HLA I complex. A) The heterodimeric structure of HLA I (MHC I) consists of type I transmembrane heavy chain (subunits α1-α3) and soluble β_2 microglobulin (β_2m). Subunits α1 and α2 form the peptide binding groove of the complex. B) Intracellular antigens, such as virus proteins, are processed into peptides by the immunoproteasome, after which the peptides are transported into the endoplasmic reticulum (ER), where they are loaded into the groove of the HLA class I complex. HLA class I complexes present antigens on the cell surface to CD8+ T cells. (Modified from Kobayashi, KS 2012 Nature rev. (94))

2.6 Enterovirus – type 1 diabetes association

2.6.1 Epidemiological studies

The pathogenesis of T1D has been associated with viral infections since the 1960s. Decades of research have provided epidemiological evidence for enterovirus association to the disease, although potential association has also been proposed for rotavirus and some other viruses (95,96). Gamble and Taylor, were the first to discovered a similar seasonal peaking pattern for enteroviruses and the onset of T1D (97). They proposed for the first time that the seasonal variation of T1D (that peaks during fall months) resembles that of enterovirus infections and that these viruses may, thus, play a role in the pathogenesis of T1D. Since then, the seasonal connection has been widely studied (98). Antibodies against enteroviruses have been measured from T1D patients and non-diabetic controls in several studies. In some of these studies, but not all, a risk association was detected (99-104). Measurement of neutralizing antibodies from European populations have indicated that CV-B1 and other CV-Bs are associated with the risk of T1D (51,59).

Table 1. Summary of retrospective case-control studies showing a positive association between enteroviral RNA in the blood and T1D in different countries.

Country	**T1D cases**		**Controls**		**Reference**
	N	Pos	N	Pos	
UK	14	64%	45	4%	(105)
UK	110	27%	182	5%	(106)
France	23	42%	27	0%	(107)
France	56	38%	37	0%	(108)
Sweden	24	50%	24	0%	(109)
Australia	206	30%	160	4%	(49)
Japan	61	38%	58	3%	(110)
Netherlands	10	2%	20	0%	(111)

Many other studies also associate enteroviruses to T1D or T1D-related autoimmunity (112-114). Table 1 summarizes retrospective case-control studies performed in different countries. In these studies a positive association between enteroviruses and T1D was found.

2.6.2 Prospective studies

Prospective studies, in which initially healthy individuals are followed up, have provided evidence that link enteroviral infection to T1D. Finland was the first country where such studies were conducted, and they showed that enteroviruses were more common in children who progressed to T1D than in healthy control children (104). This observation was supported by other studies (112,115-123).

Prospective cohort studies are important for identifying the environmental determinants involved in the initiation of the beta cell destruction. A large number of children are followed in such studies until some of them develop T1D. The largest prospective studies carried out so far include The Finnish Diabetes Prediction and Prevention (DIPP) study (115,121) that has been recruiting children since 1994, The Environmental Determinants of Diabetes in the Young (TEDDY) study (96), which has recruited children in the US and Europe, The Diabetes Autoimmunity Study in the Young (DAISY) (102,123) in the US, the Environmental Triggers of Type 1 Diabetes (MIDIA) (124) in Norway and the BABYDIAB (125) in Germany. Collectively, the results from these studies have shown an association between enterovirus infections and initiation of beta cell-damaging process by detecting viruses from serial blood or stool samples and by analyzing antibodies from serum (22,115,117,123). Prospective studies have also shown that among all different enterovirus types, CV-Bs are most often linked with T1D (113,126,127). These prospective studies can provide significant insight into the temporal relationships between enterovirus infections and initiation and the progression of the beta cell-damaging process.

2.6.3 Enterovirus in tissues of type 1 diabetic patients

Coxsackievirus B4 (CV-B4) was the first enterovirus to be isolated from the pancreas of T1D patients in 1979 (128,129). In follow-up study, it was inoculated into a mouse in which it caused diabetes (128). Later on CV-B4 was also isolated from islets of a patient suffering from T1D. This virus was able to replicate in the islets of non-

diabetic controls *in vitro,* impairing glucose-stimulated insulin secretion in infected islets of T1D patients (129). So far, enteroviruses have been reported in pancreatic islets of T1D subjects more commonly than in non-diabetic controls (30,129-131). For example, enterovirus VP1 protein was found in the pancreatic islets of six recent-onset T1D patients of the DiViD study (130). Enterovirus genome was also detected in the isolated pancreatic islets (culture supernatants) of four of these patients, while all the controls were negative. In addition, studies report that the small intestine of T1D patients is more frequently positive for enteroviruses compared to non-diabetic controls (29,132).

2.6.4 Role of pancreas studies in type 1 diabetes research

Pancreas is a multicellular organelle, and therefore it is important to establish which cell types are infected by enteroviruses. IHC studies have shown that enterovirus proteins are mainly detected in the pancreatic islets, and almost exclusively in insulin-producing beta cells (133).

Due to the difficult location of the pancreas, pancreatic samples are not usually available from living T1D patients. Instead, most of the pancreas samples analyzed from T1D patients have been obtained from either brain-dead organ donors or in routine autopsies. Post-mortem samples are not ideal, since they may have been affected by post-mortem changes and conditions that may have an effect on several markers that have been analyzed in the samples (134). The availability of pancreatic biopsies from living patients is scarce. Altogether, large tissue collections, such as the archival collection of postmortem samples from the UK (135), the nPOD (network for Pancreatic Organ Donors with Diabetes, USA) (136) and DiViD (The Diabetes Virus Detection study, Norway) (137), have made pancreas and other tissue samples available for research purposes, enabling international studies on the complex mechanisms involved in the T1D pathogenesis. DiViD is a unique study from Norway, where pancreatic biopsies were taken from six recent onset adult T1D patients. This study has given the unique possibility to study pancreatic samples soon after the actual start of the disease (3 to 9 weeks after onset), which provides insight into the pancreatic immunopathology at the time of commencement of the disease. The nPOD and UK cohorts (135,136) offer a great opportunity for pancreatic studies and have so far revealed new data on many aspects of T1D, such as the T1D –enterovirus association (30,130,131,138-140). They have also confirmed some of

the previous findings related to T1D, such as the presence or phenomenon of insulitis and the general pancreas pathology in T1D patients (90,141-143).

2.6.5 Immunological protein markers of virus infection

Several type I IFN-stimulated markers, or indirect virus-related markers, are associated with T1D. Type I IFNs are produced upon viral infection, replication, and/or the introduction of double-stranded RNA (Fig. 9). They trigger antiviral activity and induce maturation of effector T cells (144). For example, the cytokine IFN-α, a member of the type I IFN family, has been found to be expressed in the islets of T1D patients (145,146). Followed by viral infection, type I IFNs are secreted to neighboring cells and function by inducing different IFN-stimulated genes (ISGs) (147) (Fig. 9). *In vitro* studies have shown that enterovirus infection in cultured islets leads to the secretion of a number of ISGs, such as CXCL10, OAS, MDA5 (148-150), PKR and MxA (151), however, the chemokine CXCL10 production also seems to vary between the islet donors (152).

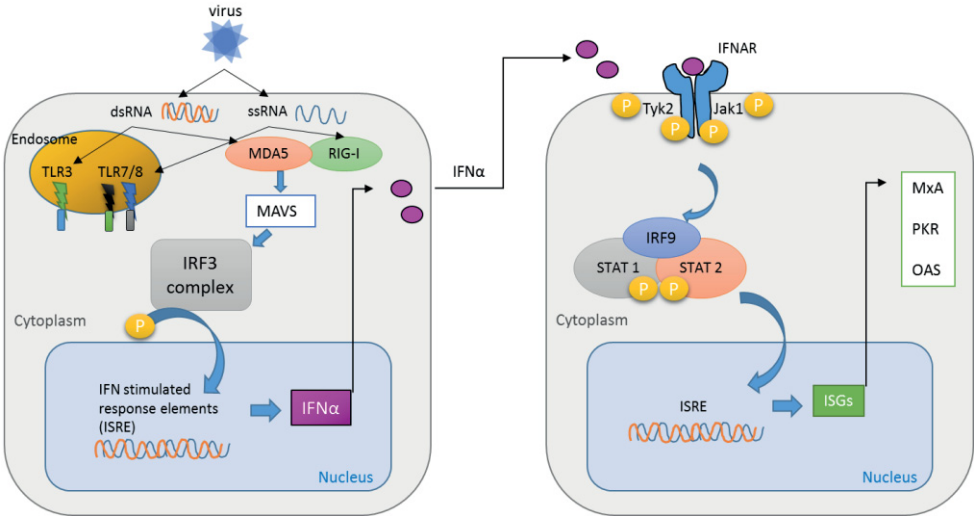

Figure 9. Schematic and simplified drawing of type I IFN production upon viral infection. When virus enters the cell, it is recognized by pathogen recognition receptors, such as TLRs and MDA5, which initiate the signaling cascade leading to activation of IFNα producing genes. Production of IFNα leads to the synthesis of ISGs and activates antiviral response in infected and neighboring cells.

PKR, MxA and other type I IFN-stimulated proteins have also been reported to be upregulated in T1D pancreatic islets *in vivo*, particularly in islets with enterovirus VP1 positivity, as well as in enterovirus-infected human beta cell line (133,153-156). MxA is a cytoplasmic protein with intrinsic antiviral properties. MxA is considered to be an important component of the early innate immune defense in humans (157). PKR, however, is constitutively expressed in mammalian cells and has intrinsic properties as the first line defense mechanism against infection and as a cell growth regulator. PKR phosphorylates eLF-2α as a result of viral infection, which ultimately results in general inhibition of translation. This phosphorylation also inhibits viral replication. PKR has an ability to exert this antiviral activity on a wide spectrum of DNA and RNA viruses (158). ISGs are also markedly enhanced in insulitic islets of living donors with recent-onset T1D (156).

IFN-α expression has also been associated with hyperexpression of HLA class I molecules in human islets (145,159). HLA I pathway has an important role in alerting the immune system to virally infected cells. Hyperexpression of class I HLA on islet cells has recently been determined to be a defining feature in T1D (160). HLA I hyperexpression has been reported in enterovirus-positive, insulin-containing islets of T1D subjects but not in non-diabetic controls, regardless of viral presence. Studies carried out in isolated human islets and human beta cell lines show that proteins involved in antigen presentation, such as HLA-C, and proteins involved in the transport of antigens, such as TAP1 (Fig. 8 B), which is associated with the HLA class I, were strongly increased upon infection with CV-B4 (154,155). TAP1 is also significantly increased in ICIs of T1D donors, correlating with increased class I HLA expression (161). It has been suggested that type I IFNs may be the key links between genetic susceptibility and environmental risk factors in T1D onset and progression (78). Furthermore, the frequency of CAR expression, the receptor for CV-Bs, has been reported to be increased in pancreatic islets in T1D and autoantibody-positive (AAb+) subjects compared to non-diabetic subjects (162,163).

2.6.6 Murine and cell models

Murine models have been used widely to analyze various aspects of T1D, including the possible connection to enteroviruses. The NOD mice are one of the most used mouse strains in T1D studies. These mice spontaneously develop autoimmune T1D, starting around 12 weeks after birth. As a model for a human disease, mouse is not optimal. For example, one of the major differences between human and NOD

mouse pancreas is the site of virus replication; enteroviruses do not strongly infect the pancreatic islets in mice but replicate in the exocrine part of the pancreas, whereas many enteroviruses have a tropism to the beta cells in humans (129,164,165). Another mouse model, the family of suppressors of cytokine-signaling (SOCS) transgenic mouse, lacks the interferon response in beta cells, due to SOCS-1 and, thus, develops a robust infection in the islets and beta cells upon viral infection. This infection is followed by hyperglycemia and loss of beta cells (166). Whereas some murine studies have suggested that enterovirus infections are linked to the development of T1D by accelerating the onset of the disease (167), other studies suggest that enteroviruses may have a contrary impact - protection from the disease (168). Studies have revealed, for example, that the virus protects the mice from developing autoimmune T1D if a CV-B is introduced into NOD mice at young age. (168,169). However, the NOD mice rapidly develop T1D if the enterovirus is introduced to NOD mice at an older age (169). The effect of the virus in the onset of T1D seems to depend on timing of the infection, virus strain and dose. Encephalomyocarditis (EMC) virus is a murine enterovirus that has also been used in T1D studies. The mechanism is dependent on the dose of the virus; higher titers lead to rapid development of T1D, due to the direct impact of cell destruction by viral replication, whereas lower titers cause the recruitment of macrophages, which in turn produce soluble mediators that eventually destroy the beta cells (167).

Isolated human islets have also been used to analyze enterovirus tropism and islet response to enterovirus infection. Enteroviruses have been reported to cause both lytic and persistent infections in such islets (170-174). They have also been shown to affect the insulin production and the organelles involved in insulin secretion but not the glucagon production (152,175). The islet cell models support the idea that different enterovirus types and strains have different capacity to induce immune response in the islets and to destroy beta cells. However, most of the studies have been carried out using species B enteroviruses, and it is not known whether these viruses differ from other enterovirus species for their ability to damage pancreatic islets *in vitro.*

Human beta cell lines have recently become available. One of the most widely used is the EndoC-βH1 cell line, an immortalized clonal human beta cell line that expresses monotypic, insulin-producing beta cells without paracrine effects of other cell types during infection. In a recent study by Nyalwidhe et al. (155) CV-B4 was used to infect EndoC-βH1 cells in parallel with human islet preparations, and viral-modified proteins were analyzed 48h post infection by quantitative MS. It was shown that infection with CV-B4 induced a reduction in insulin, increased proteins

related to antigen-presentation, and that IFN-signaling was the most increased canonical pathway identified.

Persistent or chronic enterovirus infection models have been established in pancreatic cell lines. Sane et al. established pancreatic ductal cell line (PANC-1), which was infected with a strain of CV-B4 (176). The virus was able to persist in the cell line, causing alterations such as the impairment of Pdx1, which is a transcription factor required for the formation of endocrine pancreas (176). The virus replicates in proportion of cultured cells without overwhelming cytopathic effect (so-called carrier state persistence). One of the suggested hypotheses is that enteroviruses cause a persistent infection in pancreatic islets in T1D, causing alterations on the host cell and possibly mounting the IFN response over time, ultimately by the help of acute infection episodes leading to the onset of disease (177-179).

3 AIMS OF THE STUDY

The principal aim of this study was to assess different methods for the detection of enteroviruses in cell and tissue samples, and to use these methods to investigate if enteroviruses can be detected in pancreas and other tissues of type 1 diabetic subjects. The specific objectives of this thesis were the following:

1. To develop a type- and group-targeted method for the detection of enteroviruses in formalin-fixed, paraffin-embedded (FFPE) tissue samples using QuantiGene ViewRNA *in situ* hybridization technology (I)

2. To compare the relative sensitivities of RT-PCR, immunohistochemistry, *in situ* hybridization and proteomics for the detection of enteroviruses in infected FFPE cell culture samples (II)

3. To study the presence of enteroviruses in pancreas, duodenum, spleen and pancreatic lymph node samples obtained from type 1 diabetic, prediabetic and non-diabetic subjects (III) by using immunohistochemistry and RT-qPCR

4 MATERIALS AND METHODS

4.1 Viruses and cell lines (I, II)

Enteroviruses used in studies I and II included 21 enterovirus types, 30 CV-B1 strains and 9 CV-B3 strains. The cell lines used in I and II were green monkey kidney cells (GMK and Vero), human cervix carcinoma epithelial cells (HeLa), human lung carcinoma alveolar basal epithelial cells (A549) and human rhabdomyosarcoma cells (RD).

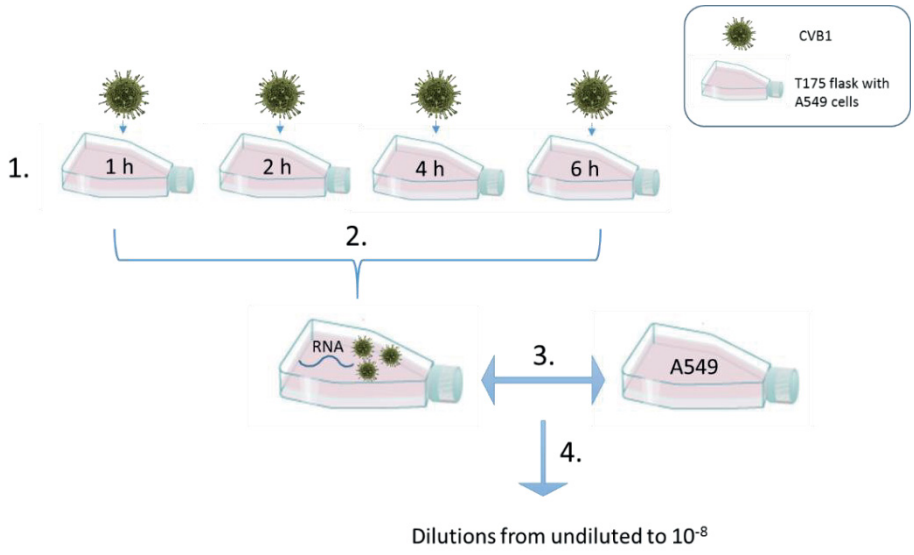

Figure 10. Schematic presentation of preparing limited dilution series**:** 1. A549 cells were grown in monolayers in T175 flasks and infected with high MOI (multiplicity of infection) of CV-B1. 2. Infections were stopped at four different time points and cells from different time points were pooled together to obtain viruses in different phases of the replication cycle. 3. The pool of infected cells were combined with non-infected A549 cells to produce a dilution series ranging from undiluted sample to dilution 10^{-8} (4.)

In I, cell lines were grown in a monolayer in complete media with 5% FBS and subsequently infected with selected viruses in their individual T75 flasks, until 50%

cytopathic effect was reached. Cells were harvested by scraping and fixed in 4% formaldehyde in PBS for 24-72h prior to dehydration and paraffin embedding.

In order to establish a dilution series of enterovirus-infected cells, in II, A549 cells were grown in monolayers in Nutrient Mixture F-12 Ham, N 6658 (Sigma-Aldrich®) medium in T175 bottles and infected with CV-B1 (an ATCC strain) and cell samples were collected by scraping 1 h, 2 h, 4 h, and 6 h post infection. The cells from different time points of infection were pooled after mechanical detachment, washed with the growth medium and immediately combined with uninfected A549 cells to produce a dilution series ranging from 10^{-1} to 10^{-8} (Fig 10). Each dilution aliquot was further divided into ten sub-aliquots and fixed or frozen according to the method applied.

4.2 Cell microarrays (I, II)

Four different cell microarrays (CMAs) were produced from paraffin blocks, which had been infected with different enteroviruses, and used in IHC and ISH. Briefly, cylindrical punches (diameter 1 mm) were drilled from individual paraffin-embedded sample blocks and inserted into new CMA recipient paraffin blocks using TMA Master (3D Histech Kft, Hungary). Four different CMAs, named EV CMA, CVB1 CMA, CVB3 CMA and Limited dilution series (Table 2, Fig. 11), were created (I and II). 5 µm-thick sections were cut from the recipient blocks and placed onto microscopic slides for histological staining.

Table 2. Overview of the CMAs created for probe set and antibody testings (I,II).

CMA	Viruses	Cell line*
EV CMA	21 enterovirus types, Adeno C, HPeV1	A549, Vero, HeLa, RD, GMK
CVB1 CMA	31 CV-B1 strains, echovirus 3	A549
CVB3 CMA	9 CV-B3 strains, echovirus 3	A549
Limited dilution series	CV-B1 ATCC strain	A549

*A549: human alveolar basal epithelial cell line; Vero and GMK: green monkey kidney cell lines; HeLa: human cervix carcinoma epithelial cell line; RD: human rhabdomyosarcoma cell line. HPeV1: human parechovirus 1

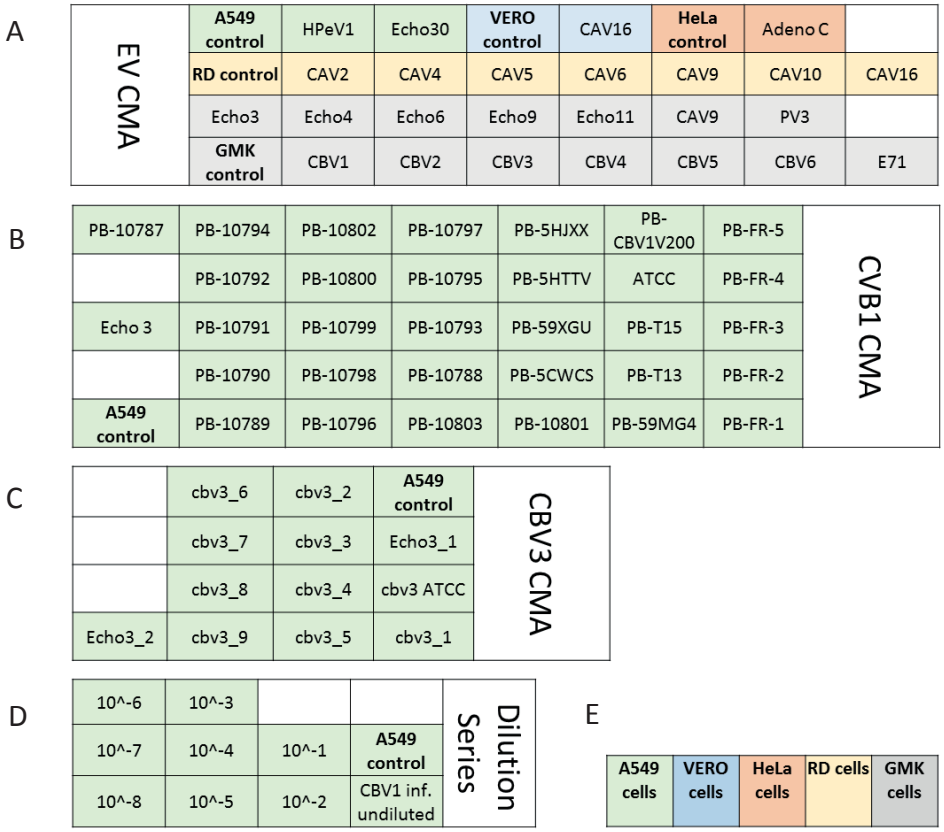

Figure 11. Schematical presentation of the different FFPE CMAs created for the probe set and antibody testings. A) EV CMA, which includes 21 enterovirus types and two non-enteroviruses on six different cell lines; B) CVB1 CMA includes 31 different wildtype CV-B1 strains and echovirus 3 on A549 cells; C) CVB3 CMA has 10 different wildtype CV-B3 strains and echovirus 3 on A549 cells; D) Limited dilution series, which includes a prototype CV-B1 infected undiluted sample, eight dilutions of it and a non-infected control sample; E) represents the color codings of different cell lines used for the CMAs. Wildtype and prototype status of the viruses used in EV CMA are detailed in I, Supplementary Table 2.

4.3 Murine samples (I)

NOD and C57BL/6J Rag$^{-/-}$ (B6 Rag-/-) mice used in the study were bred and housed in specific pathogen-free animal facilities at Karolinska Institutet, Stockholm, Sweden (in collaboration with professor Malin Flodström-Tullberg). Mice were infected intra-peritoneally (i.p.) with different types and doses of CV-Bs diluted in

200 µl RPMI medium. Organs were harvested between 3 and 7 days post infection, and fixed in 4% formaldehyde in PBS for 24 h prior to dehydration and paraffin embedding. For histology, 5 µm thick sections were cut on microscopic slides.

4.4 Human tissue samples (III)

Human tissue samples were obtained from nPOD (network for Pancreatic Organ Donors with Diabetes) project (https://www.jdrfnpod.org) –funded by JDRF (Juvenile Diabetes Research Foundation). The JDRF nPOD program recovers transplantation-quality pancreas and other organ samples from T1D donors, non-diabetic donors with and without islet autoantibodies and from donors with T2D or other conditions throughout the USA. The JDRF nPOD distributes these organs to investigators in the T1D field, who aim at obtaining information about the pathophysiological process of beta cell destruction, the etiology of the disease and in the end, who are seeking methods for disease prevention and reversal strategies (180).

Pancreas, duodenum, spleen and pancreatic lymph node (PLN) samples from T1D, T1D-related autoantibody-positive (AAb+) and non-diabetic organ donors were obtained from the JDRF nPOD collection. FFPE samples were used for immunohistochemical studies and frozen unfixed samples for RT-qPCR analyses. Tables 3 and 4 summarize the numbers and details of the sample types used.

Table 3. Number of nPOD organ donors and FFPE samples analyzed by immunohistochemistry in III.

Tissue	T1D N=64	AAb+ N=19	Control N=49	Total N of different donors per tissue N total = 132
Pancreas	64	19	49	132
Spleen	50	17	38	105
Duodenum	38	14	22	74
PLN	9	1	0	10
Total N of samples	152	50	109	311

Table 4. Number of nPOD donors and samples analysed by RT-qPCR in III.

Tissue	T1D N=41	AAb+ N=17	Control N=32	Total N of different donors per tissue N total = 90
Pancreas	33	16	29	78
Spleen	38	15	27	80
Duodenum	30	13	21	64
PLN	5	3	0	8
Total N of samples	106	47	77	230

Selected cases from T1D, AAb+ and control donors with insulin and enterovirus VP1-positive pancreatic islets were further analyzed for specific markers (see 4.6, Tables 7 and 8) to obtain information about differences between donor groups. These groups included 12 T1D cases, 7 AAb+ cases and 12 non-diabetic controls.

4.5 *In situ* hybridization (I, II)

4.5.1 Probe set design (I)

Custom probe sets for enteroviruses were designed for the QuantiGene® ViewRNA Tissue Assay (Affymetrix, Santa Clara, California, USA). Two different probe sets were designed to detect an individual enterovirus type, CV-B1 (named: CVB1 and CVB1Sub) and two for species-specific detection (named: EV AB and EV B) targeting mainly EV species A and B and EV B, respectively. The design was based either on a sequence of one viral strain (probes CVB1Sub and EV B) or on a consensus sequence of multiple viruses (probes CVB1 and EV AB). Sequences were aligned using the Clustal X program. The consensus sequence was extracted and used as a template for probe design. The probe templates were sent to Affymetrix's probe specialist, who prepared these custom probe sets.

4.5.2 Optimization of tissue pretreatment conditions

In the QuantiGene® system, the sample goes through specific pretreatment steps prior to the target probe hybridization. After removal of paraffin and alcohol dehydrations steps, the tissue is pretreated by boiling in a pretreatment solution at 95°C, followed by an incubation in a protease solution at 40°C. The boiling and protease incubation times are tissue dependent. Table 5 details the optimal times for human and mouse tissue samples and cell samples for QuantiGene® ViewRNA.

Table 5. Optimized pretreatment times (min) for boiling and protease treatment per sample type. Optimal times were also selected for multi-tissue slides, i.e.. slides containing mouse pancreas, spleen, heart and liver tissues. Some tissues were not optimized individually, and, therefore guidelines from the manufacturer are marked in the table.

Species or Sample	Tissue	Boiling time (min)	Protease incubation time (min)	Boiling and protease times (min) for multi-tissue slides
Human	Pancreas	10	10	not tested
	Duodenum	5	17	not tested
Mouse	Pancreas	5	10	10,15
	Spleen	-	-	10,15
	Heart	10*	40*	10,15
	Liver	20*	20*	10,15
Cells	-	5	10	-

*Guideline times suggested by the manufacturer

4.5.3 Workflow

ISH was performed both in Tampere virus laboratory and in a collaborative laboratory in Gainesville, Florida, US, using different commercially available methods. Sections from FFPE samples were used in both Tampere (I, II) and Gainesville (II). In Tampere, the QuantiGene® ViewRNA Tissue Assay (Affymetrix) was applied with different enterovirus-specific probe sets according to the manufacturer's instructions. Target-specific hybridization was followed by signal

amplification using specific oligonucleotides conjugated to alkaline phosphatase. Fast Red substrate was used for detection under both brightfield and fluorescent lights. In Gainesville, ISH was performed using the RNAscope 2.0 High Definition Assay (Advanced Cell Diagnostics, Hayward, California, USA) according to the manufacturer's instructions. Two probes that were specific for the whole CV-B group and CV-B3 only, were tested. Deparaffinized sections were hybridized to probes followed by amplification by serial application of amplifiers and peroxidase labels. Hybridization was visualized by DAB and brightfield light microscopy.

4.6 Immunohistochemistry (II, III)

Samples from FFPE limited dilution series (Fig.11 D) were immunostained in three different laboratories, Tampere, Exeter and Uppsala, using methods that were individually optimized in these laboratories. Primary analyses were done using a commercially available antibody (clone 5D8/1, Dako) raised against enterovirus VP1 protein. The clone 5D8/1 staining methods were previously validated between three laboratories. In addition to clone 5D8/1, polyclonal antibodies produced in rabbits against each of the viral capsid proteins VP1, VP2, VP3 and VP4 of CV-B4 Tuscany strain (GenBank database – accession no. DQ480420) were analyzed in the Tampere and Exeter laboratories, and staining was performed similarly to clone 5D8/1. Table 6 details the concentrations of the CV-B4 VP1-VP4 antibodies used in these laboratories. Antibody specificities to different enterovirus types was also tested using the CMAs (see Fig. 11) and the selected human tissue samples, which had been previously found positive for clone 5D8/1 VP1.

Table 6. Concentrations of the rabbit CV-B4 VP1-VP4 antibodies used in the IHC studies in Tampere and Exeter (II).

Laboratory	VP1A	VP1B	VP2B	VP3A	VP3B	VP4B
Tampere (FIN)	1:3000	1:4000	1:3200	1:1500	1:5000	1:2500
Exeter (UK)	1:3000	1:6000	1:3000	1:2500	1:9000	1:2000

Enterovirus VP1 stainings for human pancreas, spleen, duodenum and PLN were performed using clone 5D8/1 (III). Part of the spleen samples were also stained in

Exeter laboratory to evaluate concordance in the spleen VP1 results. Half of the pancreas sections were also stained with insulin in Tampere and half in Exeter. In the Tampere lab, the automated Ventana BenchMark LT (Ventana Medical Systems, Inc.) and the *ultra*View™ Universal detection system was used for the chromogenic stainings. In Exeter, the stainings were performed manually as previously reported (133). Table 7 summarizes the antibodies and staining conditions of chromogenic IHC.

Table 7. Antibody conditions used in the chromogenic stainings.

1°Ab	Ab details	HIER	Dilution	Incubation	2° Ab	Type of protocol	Study
VP1 Tampere	Clone 5D8/1 Dako	Tris-EDTA pH8.5	1/300	30 min, 37°C	UltraView HRP	Automated	(II, III)
VP1 Exeter	Clone 5D8/1 Dako	Citrate pH6	1/1400	1h, RT	Envision HRP	Manual	(II, III)
VP1 Uppsala	Clone 5D8/1 Dako	not available	1/2000	not available	Link 38 Dako	Automated	(II)
CV-B4 VP1-VP4 Tampere	In-house	Tris-EDTA pH8.5	Table 6.	30 min, 37°C	UltraView HRP	Automated	(II)
CV-B4 VP1-VP4 Exeter	In-house	Citrate pH6	Table 6.	1h, RT	Envision HRP	Manual	(II)
Insulin Tampere	Thermo	Tris-EDTA pH8.5	1/2000	30 min, 37°C	UltraView HRP	Automated	(III)
Insulin/ glucagon Exeter	Dako/ Abcam	Citrate pH6	1/700; 1/2000	1h RT each	HRP / AP	Manual	(III)
HLA Class I	Abcam	Citrate pH6	1/1500	45 min, RT	Envision HRP	Manual	
PKR	Abcam	Citrate pH6	1/700	O/N, +4C	Envision HRP	Manual	

Ab, antibody; HIER, heat-induced epitope retrieval; HRP, horseradish peroxidase; AP, alkaline phosphatase; RT, room temperature

IHC and IF double- and triple-stainings in the Exeter laboratory were performed together with Dr. Sarah Richardson (P.I. Noel Morgan) to study specific markers of inflammation in the pancreas of selected nPOD organ donors. Pancreas samples were stained using the following primary antibodies: enterovirus VP1 (clone 5D8/1), insulin, glucagon, PKR, and MxA. Briefly, after removal of the paraffin, the samples were dehydrated and rehydrated, followed by a heat-induced, epitope-retrieval (HIER) step. The primary antibody was incubated either 1h at RT or overnight (O/N) at 4°C, depending on the antibody. Slides were washed, followed by a corresponding secondary antibody (Alexa Fluor 488/568/633, Thermo Fisher Scientific) incubation. After wash steps, the slides were mounted and visualized using fluorescence microscope. Table 8 summarizes the details of the antibodies and staining conditions.

Table 8. Antibody conditions used in the IF stainings.

1°Ab	Ab details	HIER	Dilution	Incubation
VP1	Clone 5D8/1, Dako	Citrate pH6	1/1400	O/N at +4C
Insulin	Dako	Citrate pH6	1/700	1h at RT
Glucagon	Abcam	Citrate pH6	1/5000	1h at RT
PKR	Abcam	Citrate pH6	1/700	O/N at +4C
MxA	from O. Haller	Citrate pH6	1/500	1h at RT

Ab, antibody; HIER, heat-induced epitope retrieval; O/N, overnight; RT, room temperature

4.7 RT-qPCR (II, III)

Frozen, unfixed cell samples (II) or human organ donor samples (III) were analyzed by RT-PCR. RT-PCR analyses were performed in two different laboratories: Tampere, Finland (II, III) and Uppsala, Sweden (II).

In Tampere laboratory, RNA was extracted from 140 µl of cell sample using the Viral RNA Kit (Qiagen, Hilden, Germany). Real-time RT-qPCR, detecting the 5'UTR from enteroviral RNA, was performed as previously described (21) and briefly described here. The real-time PCR run was performed using the Quantitect Probe kit (Qiagen, Hilden, Germany) using Taqman chemistry. RT-qPCR mix was combined with QuantiTect Probe PCR Master Mix *1, forward primer 0,9 µM, reverse primer 4- 0,9 µM, probe I 0,3 µM, probe II 0,3µM, QuantiTect RT Mix 0,5 µl, Template RNA 2µl, RNase-free water up to 10µl. The primers and probes used in the method were: forward primer; CGG CCC CTG AAT GCG GCT AA, reverse primer; GAA ACA CGG ACA CCC AAA GTA, probe 1; FAM-TCT GTG GCG GAA CCG ACT A-TAMRA and probe 2; FAM-TCT GCA GCG GAA CCG ACT A-TAMRA. The cycler conditions were: 50°C 30min, 95°C 15min following by 2-step cycling up to 50 cycles 94°C 15s and 60°C 60s. In Uppsala, viral RNA was extracted from 100 µl of cell samples using RNeasy Mini kit (Qiagen). 50 ng of total RNA per sample were primed with virus-specific primers and reverse transcribed to cDNA with SuperScriptII™ RT (Invitrogen) according to the manufacturer's instructions. A semi-nested enterovirus PCR was performed in the conserved 5´region of the genome using following primers: forward primer; GCCCCTGAATGCGGCTAAT 100 (pmol/µl) Rev GATGGCCAATCCAATAGCT (100 pmol/µl) and reverse primer; ATTGTCACCATAAGCAGCCA (100 pmol/µl). Mini elute gel extraction kit (Qiagen, Sweden) was used to purify the positive PCR products that were excised from the gel (130).

In paper III, frozen human tissue samples from the nPOD collection contained pancreas, duodenum, spleen and pancreatic lymph nodes (PLNs) from T1D, AAb+ and control donors. Samples were either snap frozen, OCT tissue slabs (~50 µm slides cut from optimal cutting temperature (OCT) embedded tissue) or stored in RNALater. The tissue was homogenized with PowerLyzer® 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories, USA), and the RNA was extracted with RNeasy® Plus Universal kit. As in paper II, the presence of enterovirus RNA was analyzed after RNA extraction with real-time RT-qPCR.

4.8 Proteomics (II)

The use of proteomics technologies is a novel approach in the enterovirus detection field. The liquid chromatographic (LC) and mass spectrometry (MS) analyses in paper II were performed by a collaborative laboratory in Eastern Virginia Medical School, Virginia, USA. LC/MRM/MS/MS was targeted to recognize viral peptides

from the CV-B1-infected dilution series cell extracts. Briefly, the frozen and unfixed dilution series samples were lysed with sonication and solubilized. Aliquots of the protein samples were reduced and alkylated, after which proteins were digested with trypsin to generate peptides. The peptides were purified and concentrated to dryness prior to LC/MRM/MS/MS. The tryptic peptides were analyzed in a mass spectrometer, and FASTA-formatted virus protein sequences were uploaded into Skyline software to predict signature peptides for CV-B1 peptides. A CV-B1 2C protein peptide with sequence SVATNLIGR was selected for subsequent analysis and quantification based on the high intensities of both the precursor ion (Q1 m/z) and fragment ions (Q3 m/z) and the lack of signals in the non-infected A549 cells. The tryptic peptide samples corresponding to 16 µg of sample were automatically injected onto the column, separated and analyzed by LC/MRM/MS/MS. Relative intensity-based quantitation was achieved by comparing the area under the curve for the peptide transition pairs in the extracted ion chromatograms (XIC) for each dilution step.

4.9 Microscopy (I-III)

Most of the stained slides, with the exception of the fluorescent-labeled slides, were analyzed using scanned slide images. The scanning was done with an automated Objective Imaging Surveyor virtual slide scanner (SlideStrider, Jilab, Finland), where the Digitization was performed at a resolution of 0.4 microns per pixel (using 20X PlanApochromatic microscope objective). Image data was converted to JPEG2000 format (181) and the JVSView program (http://jvsmicroscope.uta.fi/?q=jvsview) was used to open the images. The Olympus BX60 wide-field brightfield/fluorescence microscope (Olympus America, Melville, NY, USA) was used to verify the results in I-III. The fluorescent-labeled slides were analyzed using Nikon 50i Microscope (Nikon Corporation, Japan) in IF pancreas studies.

4.10 Statistical methods (III)

The statistical analyses for paper III were performed using the SPSS 22.0 program (IBM Corp., USA) for Windows. Frequency comparison was performed with the Pearson's X^2 and Fisher's exact tests.

5 RESULTS

5.1 *In situ* hybridization probe design for specific detection of enterovirus types (I)

There is a need for enterovirus type-specific detection methods that could be used in histological studies. Therefore, specific RNA -probes for selected enteroviruses were designed and used in QuantiGene® ViewRNA ISH technique. The EV AB probe recognized all tested viruses from enterovirus species A and B. The recognition spectrum of EV B probe was similar to that of EV AB probe, with the exception of poliovirus 3 (PV3) -vaccine strain, which belongs to *Enterovirus* C. EV B probe didn't recognize PV3, while EV AB did (I, Table 2). Nevertheless, the overall intensity of the staining of different virus types was stronger with the EV AB probe. Figure 12 elaborates the differences between the EV AB and EV B probe sets.

The CVB1 probe stained all but one CV-B1 strain strongly, with clear and abundant signal, but it also reacted weakly with some other CV-B types. The CVB1Sub probe gave a more narrow recognition spectrum among the tested CV-B1 strains and did not react with any other virus type (Fig. 13). Every probe gave a negative result in the mock-infected cells as well as in the human parechovirus-1 (HPeV-1) and adenovirus C infected cells (I, Table 2.). The probes were also tested in CV-B1, CV-B3, CV-B4, CV-B5 and CV-B 6 -infected mouse pancreata (I, Table 4, Fig. 2). The results were consistent with the results obtained using infected cells (Table 9).

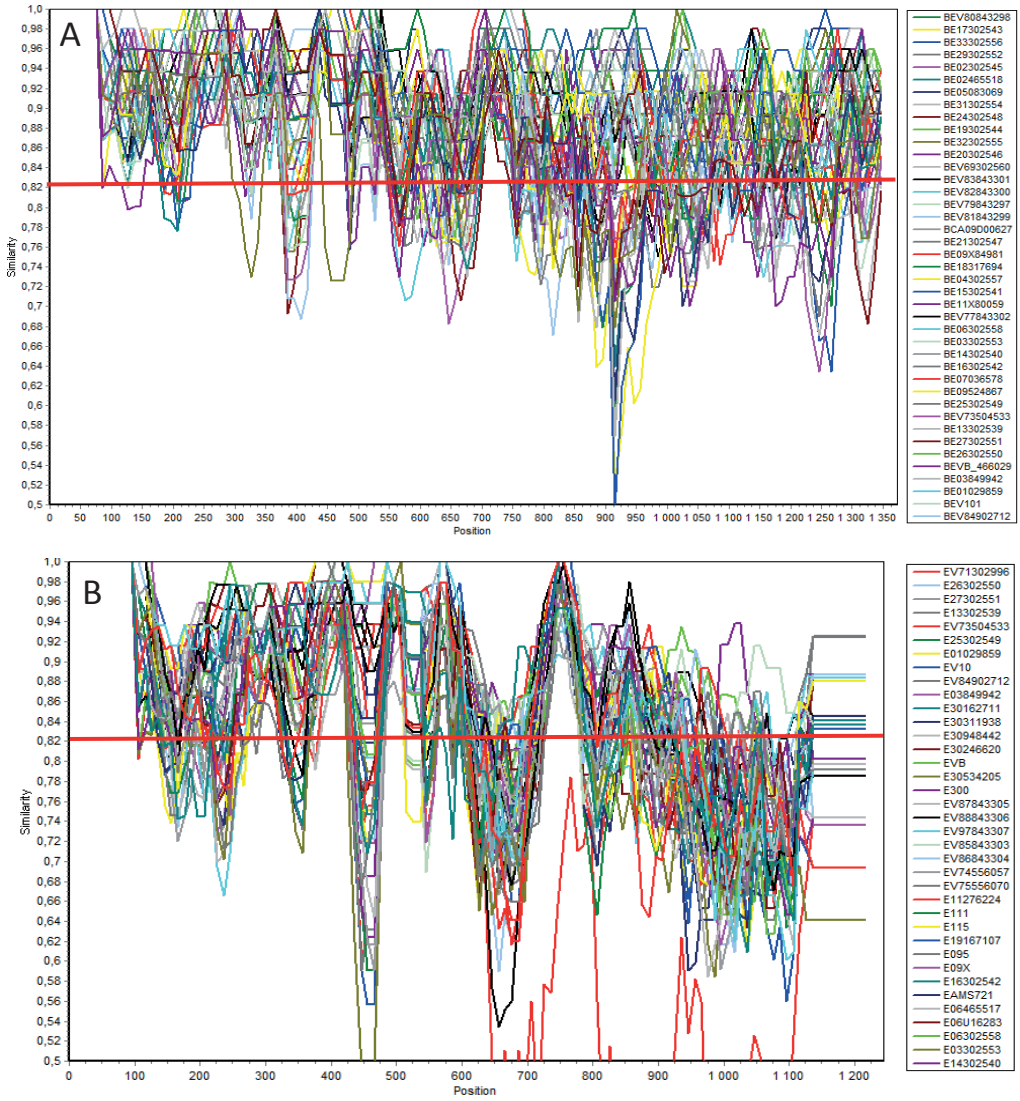

Figure 12. Example images showing the alignment results of the designed probes. A) EV AB and B) EV B probe sets. Sequences of different enterovirus strains are shown in colors. The red line marks the estimated 80-85% coverage (here 82%) that the probe set requires to recognize its target. This figure shows that EV AB has a much greater coverage of different enterovirus strains compared to EV B, since more EV AB sequences are above the threshold line compared to EV B. Y-axis shows the similarity and X-axis the nucleotide position on the probe set.

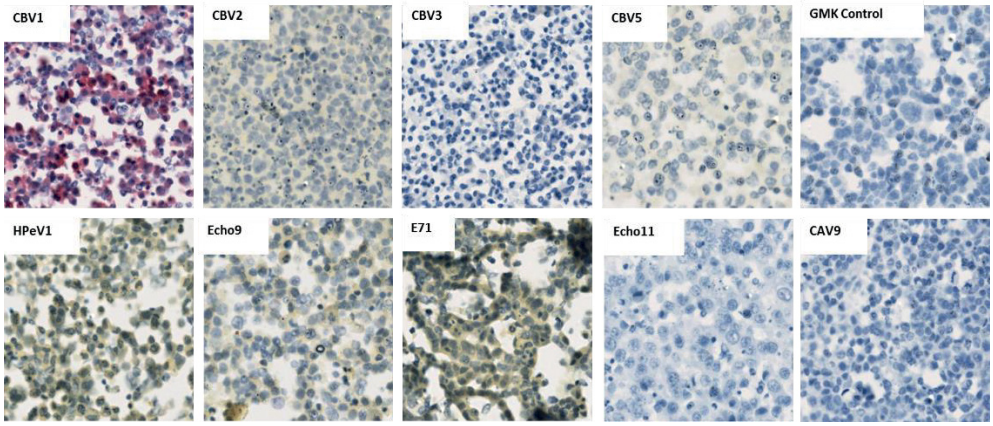

Figure 13. Specificity of CVB1Sub probe on GMK cells infected with different enterovirus types. The probe recognized only CV-B1 (positive signal indicated in red color) and none of the other enterovirus types tested. GMK control and HPeV-1 were also negative.

Table 9. Enterovirus positivity of the probe set in different CMAs and infected mice. The two widely recognizing probes, EV AB and EV B, detected (almost) all tested enterovirus types from *Enterovirus* A, B and C while type-targeted probes had a more narrow recognition spectrum.

Sample	**Enterovirus species**	**Detected enterovirus types (n/n)**			
		EV AB	EV B	CVB1	CVB1Sub
EV CMA	A, B and C	20/21	19/21	4/21	1/21
CVB1 CMA	B	31/31	27/31	30/31	14/31
CV-B -infected mice	B	5/5	5/5	3/5	1/5

5.2 Co-staining for enterovirus and insulin in human and mice pancreas using *in situ* hybridization

In order to see whether enterovirus RNA can be located to insulin-producing beta cells of the human pancreas, a method for co-staining insulin and enterovirus, was developed with the ISH (QuantiGene® ViewRNA, Affymetrix) system. Infected mouse pancreas and human pancreas with IHC-confirmed enterovirus VP1 positivity was used for the purpose. The co-staining worked well in infected mouse pancreas,

where insulin and enterovirus were not expected to colocalize, giving clear signals from both targets, insulin within the islets and enteroviruses within the exocrine pancreas (Fig. 14 C and D).

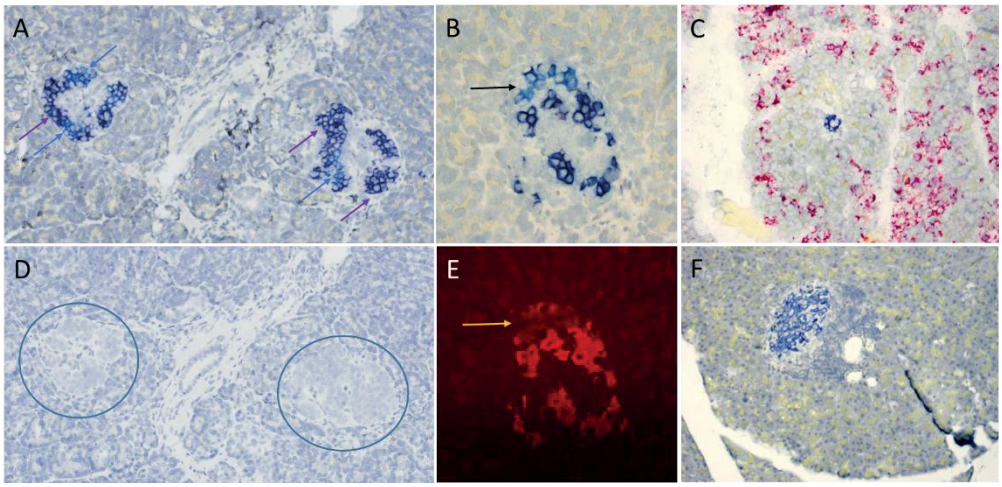

Figure 14. Co-immunstaining of insulin and enterovirus in human and mouse pancreas. ISH staining of insulin (blue; A, B, C) and enterovirus (red; D, E, F) in human (A, B, D, E) and mouse (C, F) pancreas. Co-localization of the two targets in human pancreatic islets turned the signal into purple (A and B, purple arrows and color). In E fluorescence image of the stained islet shows strong enterovirus signal. No viral signal is observed in the region where only blue insulin color is seen on the brightfield image (arrows in B and E). In D, single staining of enterovirus probe shows no signal within the same islets (encircled) of the adjacent slide of A. In C, clear enterovirus signal was seen in the exocrine tissue of mouse pancreas. A small islet is also stained with insulin in the middle. F shows insulin staining in non-infected mouse pancreas.

Instead, different results were obtained using human pancreas, where the two targets are expected to co-localize. Co-localization of the two targets was observed in some of the islets. Co-localization showed in purple in brightfield visualization, as the two signals, red for virus and blue for insulin, merged (Fig 14 A and B). When looking at the channel with a viral signal only, it completely matched the purple pattern seen in brightfield of these islets. An adjacent pancreatic section was stained with enterovirus probe only to confirm the results, but no viral signal was observed in any of the islets. This was regardless of the fact that signal was observed in the adjacent slide in co-staining of enterovirus and insulin (Fig. 14 A and D). In addition, these particular islets were also confirmed enterovirus VP1-positive by IHC in the adjacent section. None of the tested human pancreatic sections were positive for

enterovirus RNA when only the viral probe was used. Signal was seen co-localizing with insulin in some islets in co-stainings with insulin. As enterovirus-positive signal in human pancreas was seen only in co-staining with insulin but not with enterovirus probe only, it was decided that the results were inconclusive. Therefore, the enterovirus ISH technique was not used for staining the human pancreas tissue in III.

The enterovirus (EV B) probe set was also used alone to locate the viral RNA in a set of human pancreas samples from the nPOD collection. No clear positive signal was found in any of the pancreas samples (data not shown), consistent with the results presented in Fig. 14 D.

5.3 Relative sensitivity of different enterovirus detection methods (II)

To test the relative sensitivity of different enterovirus detection methods, A549 cells were infected with CV-B1 and diluted with non-infected A549 cells in a serial manner ranging from undiluted infected cell suspension to a dilution 10^{-8}. All methods tested were able to detect CV-B1 in infected A549 cells but gave no signal in uninfected cells. However, depending on the method used, the detection limit varied across a range from 10^{-4} to 10^{-8} (dilution factor of infected to uninfected cells). RT-qPCR was the most sensitive method and was capable of detecting the most diluted virus sample in one of the test laboratories. The second laboratory achieved almost the same sensitivity using a different RT-PCR method. LC/MRM/MS/MS demonstrated high sensitivity and was also able to specifically identify virus peptides in extracts of infected A549 cells. IHC reached detection limit of dilutions between 10^{-3} and 10^{-6}, depending on the laboratory and antibody used. The most sensitive IHC method was based on the commercial antibody clone 5D8/1 (Dako). Both of the ISH methods performed equally well, reaching dilution 10^{-4}, regardless of the probe used. Table 10 summarizes the sensitivities of the different methods.

Table 10. Comparison of the sensitivity of different methodologies to detect CV-B1 in A549 cells. (Modified from II, Table 1)

Method	Highest dilution reached	Target
RT-qPCR (frozen cells)		
semi-nested (Uppsala)	10^{-8}	5'UTR
real-time (Tampere)	10^{-7}	5'UTR
Proteomics (frozen cells)		
LC/MRM/MS/MS	10^{-7}	
MRM	10^{-7}	
IHC (FFPE cells)		
anti-EV VP1 Clone 5D8/1	10^{-6}	VP1 capsid protein
anti-CVB4 VP antibodies	10^{-4}	VP1, VP2, VP3 and VP4 capsid proteins of CVB4
ISH (FFPE cells)		
QuantiGene ViewRNA (Affymetrix)	10^{-4}	Conserved regions from genomic P1 region
RNAscope (ACD)	10^{-4}	consecutive regions from CVB3 VP3-VP1

5.4 Comparison of the recognition spectrum of enterovirus probes and antibodies

5.4.1 EV AB probe and anti-enterovirus VP1 clone 5D8/1 (I)

The main focus of paper I was to develop novel probe sets with different binding properties to selected enterovirus types. It also provided a good opportunity to compare the recognition spectrums between the enterovirus VP1 antibody clone 5D8/1 and the most widely recognizing probe set EV AB (i.e., protein vs. RNA detection) using the EV CMA. As a result, both EV AB and clone 5D8/1 recognized the same enterovirus types but the intensity of staining varied depending on the

enterovirus type (Table 11). The main difference was observed in the recognition of viruses from EV A species. Clone 5D8/1 was able to bind to most *Enterovirus* As only weakly, whereas EV AB showed a much stronger reaction towards *Enterovirus* As, with the exception of CV-A5, and was binding well to almost all tested enterovirus types. EV AB also overcame clone 5D8/1 in the recognition of selected echoviruses by giving a stronger signal by wider distribution in the infected sample. Both were also able to recognize PV3, which represents enterovirus species C, and as expected, neither one was binding to uninfected cells or to non-enteroviruses, including adenovirus C or HPeV-1. Neither EV AB nor 5D8/1 bound to EV71, which was the only tested enterovirus type that remained undetected in this study.

Table 11. Comparison of the ability of EV AB probe set and anti-VP1 clone 5D8/1 to recognize different enterovirus types, on a scale from – (negative) to +++ (strong positive).

Virus		Probe set	Anti-VP1 antibody
Species	**type**	**EV AB**	**Clone 5D8/1**
EV B	CV-B1	+++	+++
EV B	CV-B2	+++	+++
EV B	CV-B3	+++	+++
EV B	CV-B4	+++	+++
EV B	CV-B5	+++	+++
EV B	CV-B6	+++	++
EV B	E-3	+++	+
EV B	E-4	+++	+
EV B	E-6	+++	+++
EV B	E-9	+++	++
EV B	E-11	+++	+
EV B	E-30	+++	+++
EV B	CV-A9	+++	+++
EV A	CV-A2	++	+
EV A	CV-A4	++	+
EV A	CV-A5	+	++
EV A	CV-A6	++	+
EV A	CV-A10	+++	+
EV A	CV-A16	+++	+
EV A	EV-A71	-	-
EV C	PV-3	++	++
Adenovirus	VR846	-	-
HPeV	HPeV1	-	-

EV, enterovirus; CV, coxsackievirus; E, echovirus; HPeV, human parechovirus

5.4.2 Anti-CV-B4 VP1-VP4 antibodies

Polyclonal antibodies produced in rabbits against the viral capsid proteins VP1, VP2, VP3 and VP4 of purified CV-B4 were tested on different CMAs and selected human pancreas and duodenum samples that had been stained VP1-positive with the clone 5D8/1. All polyclonal antibodies recognized viruses from enterovirus species B, although a slight variation between the antibodies could be observed (II, Fig. 3). All viruses from enterovirus species A, and the non-enteroviruses were negative with these VP1-VP3 antibodies; however, VP4B antibody was able to recognize CV-A4 and CV-A6 weakly. In contrast, the clone 5D8/1 bound to all tested enterovirus A types weakly. Minor unspecific staining was observed in GMK control cell with every rabbit VP1-VP4 antibodies and to RD control cells with clone 5D8/1. Overall, among the tested rabbit antibodies, antibodies against VP3 capsid protein gave the best performance in terms of recognition spectrum and signal strength. According to the limited dilution series, however, the sensitivity of these CV-B4 VP1-VP4 antibodies did not reach the sensitivity of clone 5D8/1. Also, staining an adjacent slide from a strongly VP1 (clone 5D8/1) positive human duodenum and pancreas, did not provide any positivity with VP3B, also suggesting a lesser sensitivity compared to the clone 5D8/1.

5.5 Analyses of tissue samples of type 1 diabetic patients (III)

5.5.1 Insulin and glucagon in FFPE tissue samples (III)

In paper III, the insulin status of the adjacent section to the slide stained with anti-enterovirus VP1 antibody was analyzed from 132 donors (64 T1D, 19 AAb+, 49 non-diabetic controls). All non-diabetic and AAb+ donors were positive for insulin in IHC, whereas only 42% (N=27) of T1D donors showed insulin-containing islets or residual insulin-positive cells in the pancreas. Insulin positivity with respect to T1D duration showed a decreasing trend with longer disease duration (p=0.011; Fig. 15).

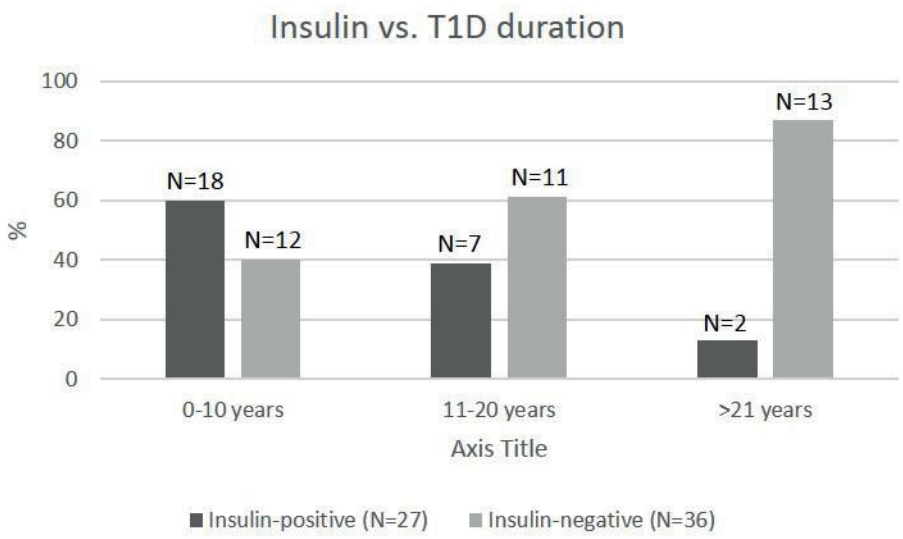

Figure 15. Insulin positivity decreased with increasing T1D duration. With T1D duration 0-10 years, 60% of donors still had residual insulin-positive cells, whereas with more than 21 years duration only 13% (N=2) had insulin-positive cells.

Markers of inflammation and insulin status were analyzed in selected nPOD pancreases from T1D, AAb+ and control donor groups (see results of inflammation markers in 5.5.6). Insulin-containing islets were detected by IHC in every donor analyzed from those samples; however, T1D donors also had insulin-deficient islets to a varying extent. The insulin was also used together with glucagon to form maps of the stained pancreas slides making it possible to localize other markers within or around specific islets. Fig. 16 shows an example of this.

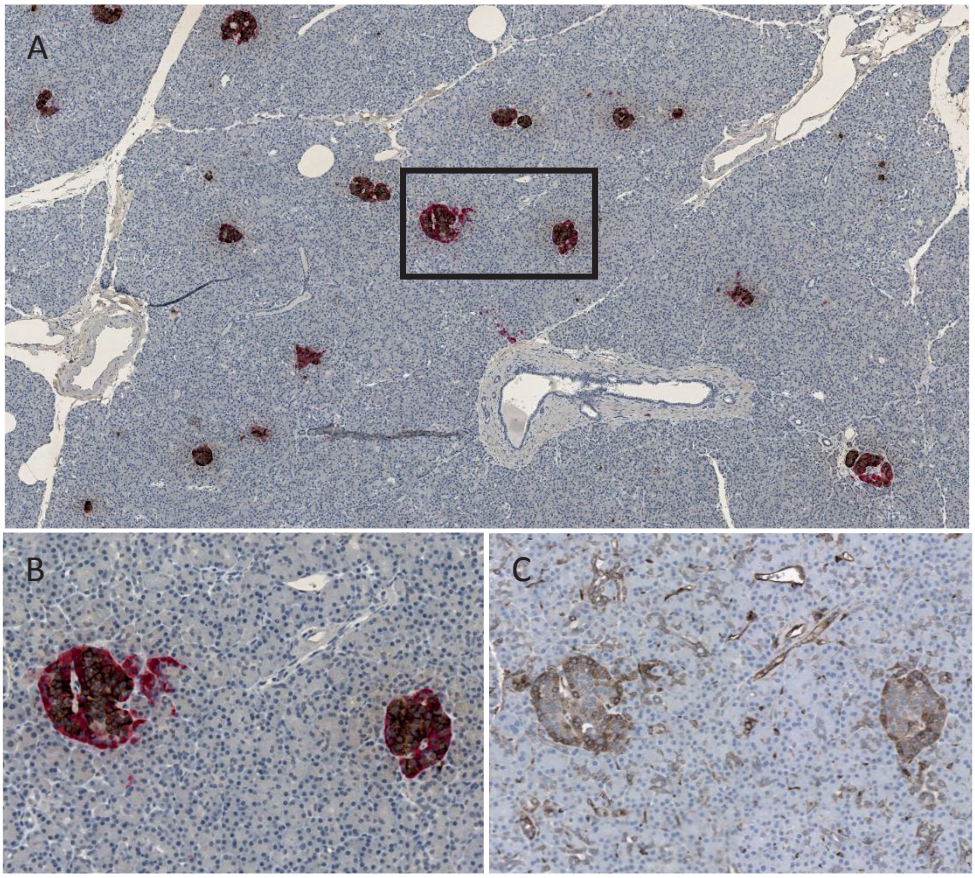

Figure 16. Images of adjacent pancreatic slides stained with insulin and glucagon. A) Insulin (brown) and glucagon (red) in an AAb+ donor. B) Rectangular area in A magnified. C) Class I HLA of the same islets in adjacent section These images represent normal expression levels of the markers.

5.5.2 Enterovirus protein in FFPE tissue samples (III)

FFPE tissue samples from altogether 132 organ donors were analyzed for the presence of enterovirus VP1 protein using clone 5D8/1. The VP1 positivity was found in the pancreas within the islets but occasionally also in the exocrine pancreas. Based on morphology, these were suspected to be ductal cells. However, no staining with specific ductal cell markers was performed. The number of VP1-positive cells per islet varied from one cell to over 20 cells and from one to hundreds of positive cells per pancreatic section. One positive cell within an islet in the entire section was considered enough to call the sample enterovirus-positive. Only islet positivity was counted as positive. T1D patients were most frequently enterovirus positive (70 %)

among the insulin-positive donors, followed by AAb+ and non-diabetic donors (53 % and 33 %, respectively, p=0.006; Table 12). Similarly to the pancreas, one or more enterovirus-positive cells in the duodenum or spleen within the entire section was counted as positive. Enterovirus VP1 in the duodenal tissue was more frequently found in T1D and AAb+ donors compared to control donors (Table 12, p=0.078). The number of VP1-positive cells varied from one positive cell to dozens per section, regardless of the donor group, and the positivity was located mostly to Brunner's glands. T1D donors tended to be more frequently VP1-positive in the spleen than AAb+ and control donors but without statistical significance (Table 12, p=0.375). The positivity usually varied from one VP1-positive cell to ten positive cells per section, and most of the positive cells were located in the spleen's follicular regions. PLNs were available only from 10 donors (nine T1D and one AAb+), and six out of ten were positive for enterovirus (Table 12). Table 12 shows the details of the VP1 positivity within each tissue of the different donor groups. Fig 17 shows typical images of enterovirus-positive samples.

Table 12. Enterovirus VP1 positivity in pancreas, spleen, duodenum and PLN of T1D, AAb+ and control donors. Table adapted from III.

	Pancreatic islets		**Spleen**		**Duodenum**		**PLN**	
	N	% positive	N	% positive	N	% positive	N	% positive
All T1D	64	42 %	50	40 %	38	40 %	9	56 %
Ins+T1D	27	70 %	23	35 %	16	38 %	4	50 %
Aab+	19	53 %	17	29 %	14	40 %	1	100 %
Control	49	33 %	38	26 %	22	14 %	0	
Total	**132**		**105**		**74**		**10**	

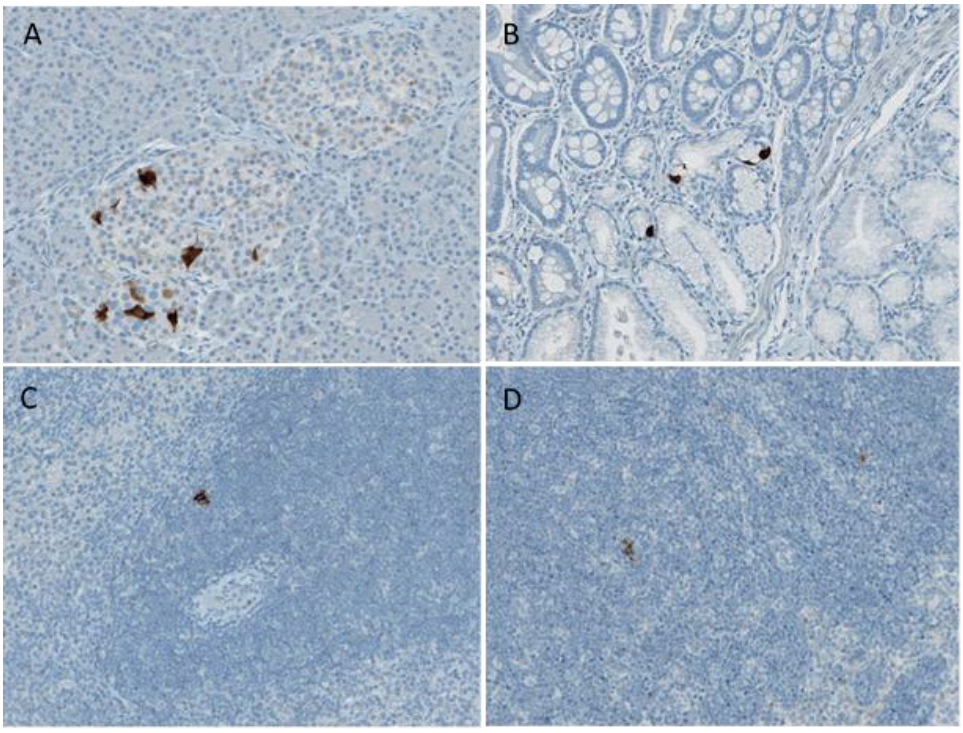

Figure 17. Enterovirus VP1-positivity (brown) in different tissues. A) pancreatic islets B) duodenum C) spleen D) PLN. The amount of positive cells can vary from one to dozens (or even hundreds) of cells per section.

5.5.3 Correlation of enterovirus positivity in different organs (III)

Tissue samples from altogether 132 organ donors were included in the study. All donors had a pancreas sample for enterovirus VP1 analysis, and most of them (N=107) also had a sample from at least one other organ. When comparing VP1 positivity in pancreatic islets of donors with residual insulin-containing islets, it was observed that 39% of T1D donors and 41% of the AAb+ donors were also enterovirus-positive in one or more other organs, compared to only 10% of the control donors (p=0.010 and p=0.012; Table 5 in III). Individual comparison of pancreas to spleen or pancreas to duodenum or PLN showed no association in enterovirus positivity; however, a negative correlation was observed in the presence of enteroviruses between pancreas and duodenum (p=0.039; data not shown).

5.5.4 Insulin and enterovirus positivity according to the duration of type 1 diabetes (III)

Of the insulin-positive T1D subjects (N=27), 70% were enterovirus VP1-positive in the islets. When evaluating the VP1 positivity in relation to T1D duration, an inverse correlation was found, since enterovirus positivity decreased with increasing disease duration (p=0.034; Fig. 18), reflecting the number of beta cells left within the pancreas section.

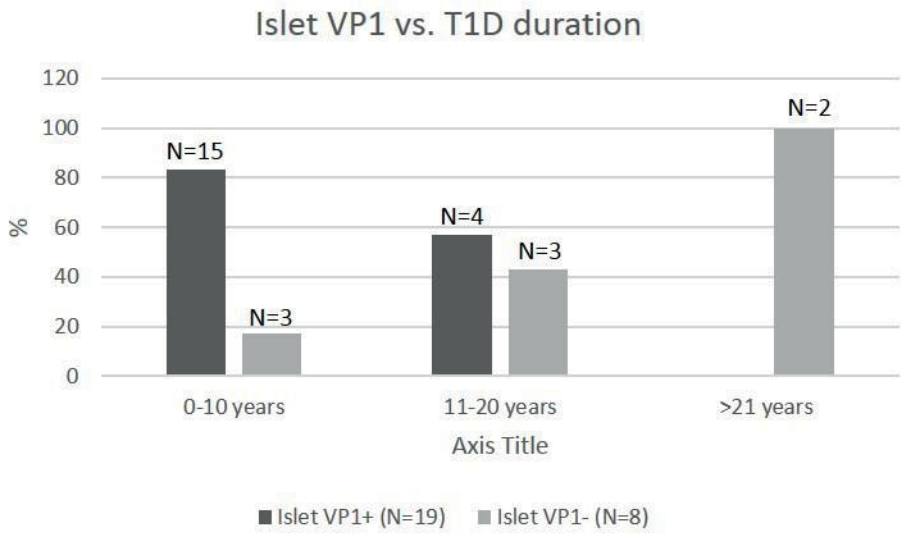

Figure 18. Enterovirus VP1 positivity decreases with increasing T1D duration. With shorter (0-10 years) duration 83% of T1D donors are VP1-positive. The VP1 positivity decreases to 57% with 11-20 years duration, and none of the donors was VP1-positive after more than 21 years of disease duration.

5.5.5 Enterovirus RNA in frozen tissue samples (III)

Altogether 231 human tissue samples were analyzed for the presence of enteroviral RNA using RT-qPCR. Enterovirus RNA was found in selected samples in all organs examined, except in duodenum (Table 13). Approximately half of the T1D donors had insulin-containing islets, and all enterovirus RNA-positive samples were found from insulin-positive donors. The aliquot type of the sample (snap frozen tissue samples, tissue sample stored in RNALater, or frozen tissue slab) had no effect on

the enterovirus positivity, except possibly with duodenum samples, which were mostly 50 µm thick tissue slabs.

Table 13. Enterovirus RNA positivity per tissue and donor type.

Tissue	N_{EV+} / N_{TOTAL}	EV positivity (%) within donor group, (N_{EV+}/ N_{TOTAL})		
		T1D	AAb+	Control
Pancreas	15/78	9 % (3/33)	50 % (8/16)	14 % (4/29)
PLN	2/8	20 % (1/5)	33 % (1/3)	No sample
Spleen	6/80	10 % (4/38)	0 % (0/15)	7 % (2/27)
Duodenum	0/64	0 % (0/30)	0 % (0/13)	0 % (0/21)

5.5.6 Immune responses during virus infection

FFPE pancreatic sections from 12 T1D, 7 AAb+ and 12 non-diabetic organ donors from the nPOD collection were analyzed for class I HLA, PKR and MxA. All donors were positive for enterovirus VP1 in the islets. The aim was to study whether the intracellular response to viral infection (measured by upregulation of PKR, in VP1-positive cells) or the intercellular responses among islet cells (measured as upregulation of class I HLA and MxA across the whole islet) differed between T1D and controls. Consecutive sections of FFPE pancreas samples were immunostained for insulin, glucagon, PKR and class I HLA. Co-immunofluorescence studies were also performed in a sequential manner on a subset of cases to assess VP1, PKR, insulin and MxA, and their possible co-localization.

As a result, PKR was selectively upregulated in enterovirus VP1-positive beta cells, regardless of the donor status (Fig. 19). A clear reduction of insulin expression was observed in the VP1+PKR+ cells in cases where multiple VP1-positive beta cells per islet were detected, which is consistent with the host protein translational arrest induced by PKR or viral proteases (Fig. 20).

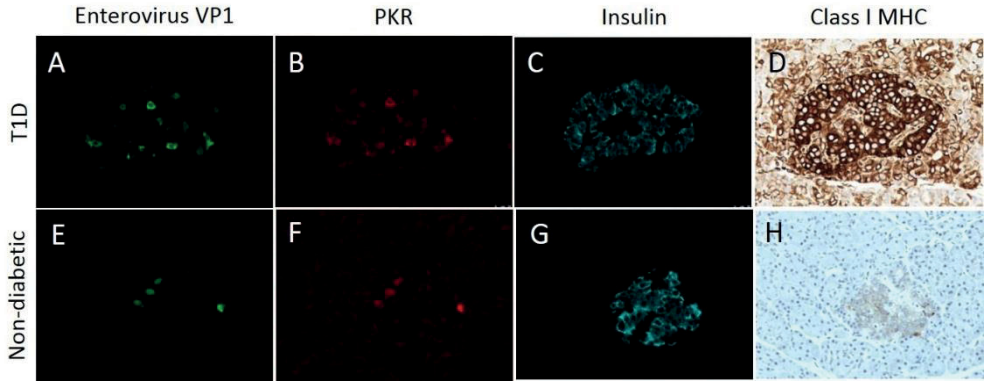

Figure 19. Class I MHC expression in VP1-positive islet of T1D and non-diabetic donor. A clear difference in the class I MHC protein expression was observed between T1D and non-diabetics. T1D donors had HLA I hyperexpression in the enterovirus VP1-positive islets (D), whereas normal levels of HLA I were observed in enterovirus VP1-positive islets of non-diabetic donors (H). PKR expression locates to VP1-positive cells in both donor groups (A, B, E and F)

Results from class I HLA stainings showed a marked difference between non-diabetic donors and subjects with T1D. Islets with enterovirus VP1 positivity hyper-expressed class I HLA molecules in T1D and a subset of AAb+ donors, whereas the islet class I HLA protein expression was normal, regardless of viral presence (Fig. 19) in non-diabetic controls.

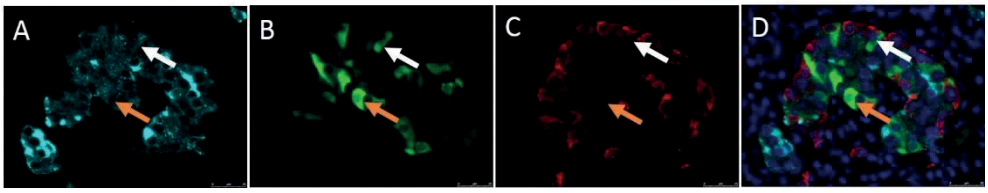

Figure 20. Reduction of insulin expression in islets with multiple enterovirus VP1-positive cells. Co-immunofluorescence staining of an islet with multiple VP1-positive cells. White arrows indicate to a VP1-positive beta cell with reduced levels of insulin. Orange arrows indicate to a strongly VP1-positive presumed beta cell, with no insulin. A) Insulin B) enterovirus VP1 C) glucagon D) merged image, nuclei stained with DAPI.

MxA protein was specifically upregulated in enterovirus VP1-positive islets of all five T1D donors and all four AAb+ donors studied. However, MxA upregulation was not observed in the VP1-positive islets of control donors (Fig. 21). The elevated MxA production was only seen in beta cells and not in alpha cells, despite the fact that both cell types hyperexpressed HLA I (data not shown).

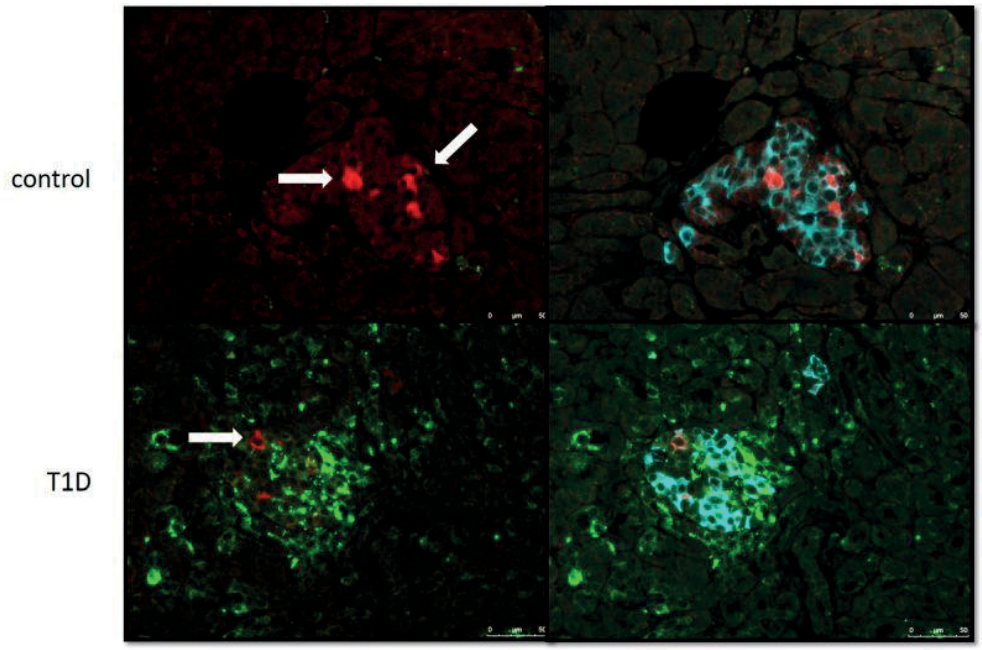

Figure 21. PKR and MxA expression in pancres of T1D and control donors. PKR (red; indicated by white arrows) was upregulated selectively in enterovirus VP1-positive islets regardless of donor status. By contrast, MxA (green) was seen in many more islets than PKR but it occurred only in islets of T1D donors (lower panels) and not in controls (upper panels). Insulin is marked with blue in the figure.

6 DISCUSSION

Enterovirus infections have been linked to T1D in several epidemiological studies but the actual mechanism leading to beta cell damage and cessation of insulin production remains unknown. It would therefore be important to study the nature of enterovirus and T1D association further. Studies addressing the tropism of enteroviruses to beta cells and possible persistence of enteroviruses in the pancreatic cells would help to understand the mechanisms of this association. In addition, development of sensitive methods for the detection of enteroviruses is needed.

Consequently, the current study had two focus points: First, the study focused on developing and optimizing methods for sensitive and specific detection of enteroviruses in cell and tissue samples. Sensitivities of these methods were also compered to each other. These tasks included the development of novel enterovirus-specific probe sets for the commercially available ISH technique. The lack of type-specific identification of enteroviruses from the pancreas of T1D patients has hindered the progress in this field, but the present study shows that ISH technology, coupled with careful optimization of probe sequences, can be used to detect enteroviruses in type-specific manner. The second focus area was to apply some of these detection methods to human tissue samples to evaluate possible presence of enteroviruses. Differences in the anti-viral response in the pancreas of T1D and control subjects were also addressed.

6.1 Custom enterovirus probe set design and detection spectrum

The first paper describes a specific RNA-based method for the detection of enteroviruses in FFPE samples. This kind of method could allow exact localization of the virus and, thus, would be useful in evaluating the role of enteroviruses in T1D pathology. The aim was to create a group and type-specific detection methods for enterovirus species A and B and for a single enterovirus type, CV-B1, that has been linked to T1D in epidemiological studies (113,126). This was enabled by careful

analysis of viral genomic sequences for the design of optimal QuantiGene® ViewRNA ISH probe sets.

The results demonstrated that the QuantiGene® ViewRNA ISH technique can be used to detect enteroviruses in FFPE samples. Different approaches were applied in the design of the probes, which also reflected the recognition spectrum of the probe targets. The most conventional approach based on a continuous sequence from a single viral strain, resulted - not surprisingly - in the narrowest enterovirus recognition. In other words, the CVB1Sub probe recognized only CV-B1s, which in that way makes it a type-specific probe. However, CVB1Sub did not recognize all the CV-B1 strains tested nor did it recognize all the CV-B1 strains in an equal manner. This is not surprising, since the target sequence covered continuous sequence from the VP1 and VP3 structural regions of one CV-B1 strain (I, Supplementary, Table 5.), while sequence variation occurs also between the strains within one single virus type. The results seemed quite logical, as the QuantiGene® ViewRNA ISH requires around 80-85% sequence coverage for the signal to be produced, i.e., approximately 80% of the target sequence has to be recognized by the probe sets (information obtained from Affymetrix).

The EV B probe was designed to recognize enteroviruses mainly from species B. Again, only one CV-B1 strain sequence was used for the design, but this time, instead of choosing a continuous sequence, only the most conserved parts from the P1 region of the genome were used. This probe showed how conserved some regions of enteroviruses are, since the probe was able to recognize all the 20 tested species B virus types. A further step was taken with the design of the EV AB probe, which recognized all tested enteroviruses from species A, B and C with better coverage and stronger staining intensity compared to EV B probe. EV AB was designed by choosing consensus sequences from multiple enterovirus types/strains from structural and non-structural regions and also by selecting 25% more sequences to be included in the probe set. The EV AB probe set was found to be the best probe set for broad-spectrum enterovirus detection with QuantiGene® ViewRNA ISH system.

This method is a great tool when the aim is to localize mRNA targets from cells and/or tissues when the target in question is abundantly present. Co-staining with two different targets that are not expected to co-localize worked also, which is an advantage that saves tissue material, reagents and time. However, co-staining of two targets that were expected to co-localize in the pancreas gave inconclusive results; thus, this technology would need further optimization if used for such

purposes. The QuantiGene® ViewRNA ISH technology is also relatively labor intensive and prone to variation in assay performance. It is important to be precise with temperatures and incubation times, which have a clear impact on the results. Conversely, the system offers some flexibility within the different steps of the protocol with careful optimization. The QuantiGene® ViewRNA ISH system is also relatively expensive to be used for large number of samples.

6.2 Relative sensitivity of different enterovirus detection methods

The sensitivity of a virus detection method is fundamentally important when it comes to detecting low-abundant targets, such as enteroviruses from human samples. As good as the QuantiGene® ViewRNA ISH is in the specificity to detect different enteroviruses, the second paper showed that its sensitivity is not optimal when the amount of the virus is low in the sample.

Punctate, intensely stained enterovirus-positive cells can be found within the pancreatic islet cells of T1D patients using an anti-enterovirus VP1 antibody (clone 5D8/1, Dako) (130,140). However, other staining methods, such as ISH or staining with other enterovirus antibodies, have failed to confirm the findings (unpublished results from nPOD and PEVNET studies).

In the second paper, a limited dilution series of enterovirus-infected cells was created to compare the sensitivity of different methods: A549 cells were infected with CV-B1 ATCC strain, and the cells were harvested at different time points post-infection to have cells that represent different stages of viral replication cycle. An FFPE cell microarray block was created for histology. Sections were cut on microscopic slides, and all ten samples (negative control, undiluted, and dilution from 10^{-1} to 10^{-8}) were stained at once on the same slide. Frozen samples for RT-PCR and proteomics were prepared from the same cell aliquots that were used for FFPE samples.

The second paper showed that, overall, all tested methods were able to detect CV-B1 from the cell samples. However, the sensitivity of these methods varied. RT-PCR and proteomics were the most sensitive methods to detect enterovirus in this setting. It was also shown that anti-enterovirus VP1 antibody (clone 5D8/1) in IHC was the most sensitive staining system tested. There was up to a two-fold log difference when compared to the commercial ISH methods used in this study. The results of the other enterovirus anti-VP antibodies showed lower sensitivity compared to ISH in this particular setting.

The proteomics LC/MRM/MS/MS approach is novel in the enterovirus detection field. In the second paper, it was the most sensitive method after RT-PCR, reaching dilution 10^{-7}. One of its advantages is that the identification and validation experiments are performed simultaneously, which ensures that the signals are derived from the same peptide and the MS/MS spectrum data can be used for protein identification in database searches. It also provides sequence information about the detected virus, which in some cases could even specify the virus type in the sample. LC/MRM/MS/MS could provide new solutions to identify enterovirus infections in chronic diseases, e.g., in pancreatic sections and in blood from patients with T1D.

This study offered a good indication of the relative sensitivities of different methods that can be used to detect enteroviruses in infected samples; however, it also has limitations. First, it is important to realize that a cell model differs from the actual clinical tissue sample where the nature of infection may be different. The infections were also performed with only one particular CV-B1 strain, which, in theory, may not be recognized equally well in the assays. However, this was most likely not an issue, since the antibodies used in IHC and the probes used in RT-PCR and ISH recognize a wide range of enteroviruses (I). The proteomics analyses also do not depend on the enterovirus type; therefore, it is likely that comparable results would be obtained with other enterovirus strains or types.

All three laboratories, which participated in this study, used the same antibodies in the IHC stainings but with different sensitivities. This could be explained by the low number of virus-positive cells present in the most diluted samples where the number of infected cells may vary from one section to another. This is because the virus-positive cells are stochastically distributed at higher dilutions. Therefore, the results could be reliable if more sections from the lower dilution samples were analyzed. It is also possible that differences in the staining protocols in each laboratory, for example, different antigen epitope retrieval approaches or manual (Exeter) versus automated staining (Tampere and Uppsala), could have an effect on the sensitivity (see Materials & methods, Table 7). Noteworthy, however, was that clone 5D8/1 was consistently the most sensitive antibody tested in each laboratory. The ISH techniques applied in two different laboratories reached the same sensitivity in both laboratories. These two commercial ISH techniques, although marketed by different companies, have the same principal and amplification technique (45). Consequently, it is logical that comparable results were obtained. PCR and proteomics were used frozen samples, whereas 5 µm thick paraffin samples were used for IHC and ISH. Therefore, the sample size in PCR and proteomics was

not comparable to that used in IHC and ISH. These limitations also apply to the clinical samples, which is why the use of more than one enterovirus detection method is recommended to reach optimal specificity and sensitivity.

6.3 Enterovirus in human tissue samples

The JDRF nPOD collection analyzed in report III is the largest study series so far used to assess enteroviral presence in tissue samples of T1D patients (cadaver organ donors with T1D). It is also one of the first studies where up to four different organs were analyzed from the same individual. What comes to the enterovirus VP1 IHC in the pancreas, this study confirmed the previous findings that the pancreatic islets of T1D subjects are more frequently enterovirus-positive compared to non-diabetic controls (30,130,131,140), even though enteroviruses were also found in the pancreas of one third of the non-diabetic controls. Like insulin-producing beta cells in T1D, the VP1 positivity decreased as the duration of the disease increased, which is not surprising since enteroviruses have a tropism to insulin-producing beta cells (182). Enterovirus VP1 positivity was also analyzed in the exocrine part of the pancreas. The positivity was usually seen in ductal-like cells (cell type was not identified by cell-specific markers), but no significant differences were observed between the donor groups (positivity rate ranged from 36% to 43 %, data not shown).

Enterovirus positivity was found also in the duodenum and spleen, and positivity rate was higher among T1D donors compared to non-diabetic controls. However, in the spleen there was only a tendency towards this direction that was not statistically significant. The duodenum, however, showed a marked difference in the enterovirus positivity of T1D and AAb+ donors compared to controls. Moreover, when comparing the enterovirus positivity between different organs, it was observed that T1D and AAb+ donors were more often enterovirus-positive in multiple organs compared to control donors. Frequent detection of enteroviruses in multiple organs in T1D patients compared to control subjects supports the idea that T1D is indeed associated with an enterovirus infection. The beta-cell specific expression of enterovirus protein together with the higher prevalence of the virus in T1D patients suggests that the infection may play a role in the pathogenesis of T1D. Conversely, it is also possible that diabetes may indirectly increase the susceptibility to enterovirus infection, e.g., by affecting the immune system. However, the fact that also the AAb+ (and still non-diabetic) subjects were more

frequently virus-positive than controls argues against this possibility. One reason for the higher enterovirus positivity rate in the T1D group could be related to the proinflammatory milieu created by the autoimmune process (65,183) which, in theory, could make the organs susceptible to the virus and facilitate its spread to multiple organs.

Interestingly, an inverse association existed between enterovirus positivity in pancreatic islets and duodenum in T1D donors, as individuals with shorter duration of T1D were more often enterovirus positive in the pancreas, whereas individuals with longer duration were more often positive in the duodenum. The higher prevalence of the virus in the duodenum of T1D and/or AAb+ donors compared to control donors is in line with the previous findings (29,132). The small intestine is the primary replication site of enteroviruses; therefore, this finding can reflect a higher frequency of acute or chronic enterovirus infections in T1D patients. However, it is also possible that the duodenal mucosa of T1D patients is abnormally permissive for enteroviruses. T1D is a multi-organ disease affecting not only the pancreas but also the gastrointestinal tract. Reports suggest that many diabetic patients suffer from gastrointestinal disorders or diabetic enteropathy, which has a largely unknown ethiopathogenesis (184). Patients may suffer from abnormalities in the gut mucosa and altered gastrointestinal motility, which can cause a variety of symptoms, and be associated with intestinal inflammation, in T1D patients. This dysfunctional intestinal homeostasis may worsen over time, perhaps making the intestine more prone to viral infections in patients with long T1D duration. However, AAb+ donors were also frequently enterovirus-positive in the duodenum, suggesting that the diabetes process per se is not critical in this phenomenon.

Enteroviruses were more common in tissues of T1D donors compared to control donors. However, these results do not directly indicate whether the virus was there before the onset of T1D or whether the tissue was more prone to infection due to the T1D associated changes. It is possible that enteroviruses infect islet beta cells in the pancreas at any age and any stage of the diabetic process, possibly even several times during the lifespan. For example, enteroviruses were frequent in the pancreas of AAb+ donors who were still non-diabetic. In addition, enterovirus infections may also accelerate the progression of islet autoimmunity as suggested by frequent detection of enteroviruses at the onset of clinical T1D in retrospective studies (114) and accelerated progression of the beta-cell damaging process in enterovirus positive children in prospective studies (123) .

As mentioned earlier, several studies have suggested that enteroviruses have tropism to beta cells. From this point of view, one of the most important findings is that enteroviruses can infect and damage beta cells and induce inflammatory infiltrates in lethal enteroviral infection in children in the absence of T1D (185). This suggests that T1D-associated changes in the pancreatic islets are not associated with virus tropism, which instead may simply be a natural biological feature of certain enteroviruses. The mechanisms of tropism may be related to the expression of viral receptors in beta cells as the main receptor of CV-B is strongly expressed by these cells (163). In addition, ability of a single cell to mount innate immune responses can significantly modulate the tropisms of enteroviruses. In fact, it has been shown that enteroviruses cause rapid beta-cell damage in transgenic mice, in which beta-cells do not produce interferons (186) and in which the innate immune system response to the virus is lower in beta-cells than in alpha cells (78). In the present study, the subjects with T1D, especially at or near the onset of diabetes, had more enterovirus VP1-positive cells per islet compared to control subjects suggesting that the virus can spread wider in the islets of T1D patients.

Interestingly, enterovirus was detected also in the spleen, even though the difference between T1D patients and controls was not as clear as in the pancreas and duodenum. The spleen is an organ that has been referred to as “the filter of the blood” due to its extensive vascularization and the presence of macrophages and dendritic cells that remove microbes and dying red blood cells from the blood. Other functions of the spleen include antibody synthesis and reservoir of blood cells (187).Thus, the presence of enteroviruses in spleen may reflect ongoing or recent infection in other organs or anatomical sites, and transport of the virus to spleen via blood, possibly by infected leukocytes. It is not known how long the virus could persist in the spleen after the acute phase of the infection. The relatively high frequency of virus-positive cases suggests that enteroviruses may stay in spleen for quite a long time.

RT-qPCR was performed on 230 different samples. These included samples from the pancreas, duodenum, spleen and PLN from 90 different donors from three different donor groups (from 41 T1D, 17 AAb+, and 32 control donors). Tissue samples were either snap-frozen or stored in OCT or RNALater. When only small amounts of tissue samples were available, a so-called tissue slab of the sample was analyzed, which in this study was approximately a 50-µm thick section cut from the frozen OCT tissue sample. The majority of the duodenum samples analyzed were tissue slabs, and duodenum was also the only organ in which this kind of samples were available. As

it turned out, all the duodenum samples were enterovirus-negative in RT-qPCR, but many of them were positive for enterovirus VP1 protein in IHC. The reason for this discrepancy is unknown, but several options can be speculated. First, when organs are collected, most of them go through the perfusion process to better preserve the integrity of the organ (188). With the nPOD samples, the intestine, however, does not go through this; therefore, it is subject to autolysis and other degrading processes. As is well known, RNA is very unstable compared to proteins, and at conditions present at organ collection, it is possible that viral RNA is degraded by numerous RNases and other degrading enzymes (189,190). Second, it is even more unlikely to find viral positivity, as many of the duodenum samples analyzed were only 50-µm thick tissue slabs, meaning dramatically smaller-sized samples and, thus, including less RNA compared to the 'normal' sized tissue piece (at least 2 mm x 2 mm). Another aspect to consider is that the analyzed samples may contain substances that inhibit PCR reaction, and thus have an effect on the assay sensitivity (191-193).

From the altogether 230 analyzed samples, 23 were positive for viral RNA (15 pancreas, 2 PLN and 6 spleen samples). Enterovirus-positive samples came from all donor groups, but the numbers were too small to make any comparisons between the groups. One T1D case was enterovirus positive in the pancreas, spleen and PLN, and another T1D case in the pancreas and spleen. All the other enterovirus-positive samples came from different donors, in other words only one organ was positive for the virus. It is also important to note that not all four organs were analyzed from every donor. Further studies are needed to verify the presence of enterovirus in these organs, and such studies are currently ongoing in the nPOD-Virus community.

The samples analyzed in RT-qPCR and IHC represent a tiny part of the whole tissue and it would therefore be optimal to analyze multiple tissue regions. Unfortunately, this is not always possible due to the limited amount of tissue material.

Although more enterovirus positivity can be found in T1D organ donor tissues compared to control donors, causality cannot be based only on staining results. Donors with diabetes-related autoantibodies but without the overt disease might be the most interesting group to study when examining the mechanisms operating before the disease onset. In this particular group the more you have the autoantibodies, the higher is the risk of the disease (73). Therefore, the best group to study would be the individuals with multiple autoantibodies. The challenge is that tissue samples are only rarely available from such cases.

6.4 Immune response to viral infection

As previously discussed, the host response to the virus may contribute to virus–induced beta cell damage and determine whether the infection leads to an autoimmune-like process in the islets (Chapter 2.5). Therefore, organ donors that were enterovirus VP1-positive in the pancreas, including donors from T1D, AAb+ and control groups, were further analyzed for the expression of selected markers that reflect the host immune responses against viral infection in the pancreas. The aim was to find out whether their expression in enterovirus VP1-positive islets differs between the donor groups. This study included altogether 31 VP1-positive organ donors (12 T1D, 7 AAb+ and 12 control donors).

The most striking difference between the donor groups was observed in class I HLA expression as all T1D donors expressed elevated levels of HLA I across the enterovirus VP1-positive islets, whereas all control donors had normal class I HLA levels in enterovirus-positive islets. This finding is in line with previous publications (143,160,194). Richardson et al described the HLA I hyperexpression as a hallmark of immunopathogenesis of T1D (160). They observed that HLA I levels were clearly elevated both at protein and RNA levels in insulin-containing islets of T1D patients. β2M- and STAT1 expression levels were also increased. In addition they observed that islet hyperexpression of HLA I molecules is detectable in many T1D patients for up to 11 years of disease duration, after which it starts to decline.

In the present study, MxA was also overexpressed in the islets of all T1D and AAb+ donors but in none of the control donors. MxA gene is IFN-regulated protein and induced by type I and type III IFNs but not by type II IFN or other cytokines. The MxA protein is considered an important component of the innate immune defense in humans (157) possessing intrinsic antiviral properties. Thus, these results clearly suggest that the local immune response is more strongly activated in the enterovirus-positive islets in T1D patients than in controls and that it involves interferon-mediated responses. This unique finding supports the concept that host response to the virus may be an important factor in virus-induced beta cell damage (78,195). Antiviral responsiveness is regulated by genetic factors, including genes, which are associated with the risk of T1D (see below). The present study showed no difference in the protein kinase R (PKR) expression between the donor groups. PKR, which is induced directly by dsRNA but also through type I IFNs, was positive in enterovirus VP1-positive cells regardless of the donor group. Increased PKR levels were associated with reduced insulin immunopositivity in individual beta cells, which is consistent with possible induction of host protein translational arrest by PKR or by viral proteases (196-198).

Based on the differences in class I HLA expression in T1D and control organ donors, it can be speculated that individuals at risk of T1D may have an inherited tendency to strong IFN responses, which amplifies inflammatory signals that ultimately lead to autoimmunity and disease development. Innate immune response pathways are partly regulated by SNPs, such as those in *TYK2* and *IFIH1* genes. Different genotypes in these SNPs could ultimately lead to either rapid disease development or offer protection from the disease (77,79,199). It has been suggested that TYK2 regulates apoptotic and proinflammatory pathways in beta cells by modulating IFN-α signaling, which subsequently increases HLA I protein expression and modulates chemokines such as CXCL10. To support this, chemokines, such as CXCL10, have been found to be increased in the pancreatic islets after enterovirus infection (148-150). Thus, it is possible that these SNPs can lead to more pronounced IFN-response in virus-infected T1D subjects. Analogously, human beta cells that have been silenced for TYK2 show decreased type I IFN pathway activation and lower production of IFN-α when exposed to dsRNA mimic polyinosinic-polycytidylic acid (PIC) (77). In addition to SNPs, the T1D-associated HLA-DR risk and protective alleles, respectively, have been shown to be associated with stronger (risk alleles) and weaker (protective alleles) immune responsiveness against enteroviruses (200). Therefore, it is possible that virus-gene interactions can also explain the observed differences in islet antiviral response in T1D patients and controls.

The present study suggests that an enterovirus infection in the pancreatic islets may contribute to the upregulation of PKR, HLA I and MxA in islets of T1D patients. However, this does not completely rule out the possibility that other viral infections are also present in the pancreas, partly causing these phenomena. PKR upregulation was always seen in cells that were enterovirus VP1-positive. In some cases, however, PKR positivity was also observed without VP1 presence. PKR is activated by dsRNA, which is produced during enterovirus replication in the infected cell. However, other viruses can also produce dsRNA. It has been reported so far that at least influenza A viruses can grow in human pancreatic cells and cause pancreatitis and diabetes in an avian animal model. In that particular study, the virus was also able to infect human pancreatic islets (201). Rotavirus infection has also been associated with pancreatic islet autoimmunity through molecular mimicry (202). Rotaviruses have also been shown to infect the pancreatic islets in various animal models (203). However, rotavirus itself has not been directly found in human pancreatic islets. In addition, some other viruses could also infect beta cells, including adenoviruses (Ads) that use CAR as their receptor for cell entry (such as Ad 2, 4, 5, 15, 19p, 31 and 41 (204); however, adenoviruses have not yet been found in the pancreas. Ad 41 has tropism to the intestine, which is anatomically closely

related to the pancreas; therefore, it can be speculated that, like enteroviruses, it may also find a route to pancreas.

6.5 Causality of EV – type 1 diabetes association

Do enteroviruses cause T1D or are they just a consequence of pathological manifestations of the disease? Do enteroviruses appear stochastically, and/or is there a yet-to-be-resolved confounding factor that has its effect on enterovirus appearance in tissue samples? These are not easy questions to answer, nor is the answer most likely a simple 'yes' or 'no'. Causality is difficult to prove, especially if intervention trials have not been carried out. Additionally, factors that are causing the disease in certain populations might not do so in all other populations. The population genetics, environmental factors and epidemiological patterns, such as demographical, biological, geographical and other factors in populations, may play a role in disease development and onset (205). Therefore, while many times results from a given population can be generalized to wider populations, one must remember that certain populations may be more susceptible to disease development than others.

As mentioned earlier, T1D is most likely a disease with multiple origins; therefore, enteroviruses may not be the causative agents in all disease forms. The cumulative evidence of a link between enteroviruses and T1D is, however, strong and repeatedly documented. Consequently, it seems likely that enteroviruses could play a role in at least part of T1D cases. This is also supported by the fact that the majority of T1D patients are positive for enterovirus in the pancreatic islets. On the other hand, the "hygiene hypothesis" suggests that the decreasing incidence of infections in developed countries could be a major driving force behind the increasing incidence of allergies and autoimmune diseases including T1D (169,206,207). Multiple approaches are needed to understand these relationships. Interventions, most obviously vaccination, could directly demonstrate the causality of infections.

6.6 Relevance of tissue studies

This doctoral thesis focuses on pancreatic studies, and more specifically on immunohistochemical stainings. Pancreatic studies, however, can be carried out using various kind of approaches. While histological stainings identify viral proteins, RT-PCR identifies the presence of viral nucleic acid, which can be further studied by sequencing the amplified genome region. Sequencing may reveal which viruses or,

more likely, which virus families are present in the tissue. Laser-captured microdissected islets can be studied to evaluate possible islet origin of the virus accompanied by other analyses such as gene expression analyses (160). The sensitivity of the assays used to detect infection in target tissue depends on the assay technology and the quality of the sample. For example, fixation of the tissue has a major effect on virus detection by immunostainings, and the details of the fixation process need to be taken into account when selecting optimal assays for enterovirus detection (e.g., fixation solution, duration of fixation, paraffin embedding). The storage condition and the expected amount of virus in the sample may also influence the results. (134,208)

As shown in paper III, enteroviruses can spread to different organs. This is in line with previous human studies and mouse models of enterovirus infection. Murine studies have shown that enteroviruses infect multiple organs and that the affected organs vary between mouse strains and the virus type in question ((209); unpublished data from nPOD-Virus community). The same applies to humans: Certain enterovirus types or strains can infect certain organs. However, it is challenging to type the virus in the tissue using histological techniques due to lack of reagents that specifically and sensitively could recognize enteroviruses from tissues in a type-specific manner. On the other hand, sequencing of the viral genome that has been amplified by RT-PCR from tissue sample can provide information about the type of enterovirus present. The sensitivity of RT-PCR is critical for the success of this approach.

Prospective cohort studies have a great advantage compared to pancreas tissue studies since they make it possible to deduce at which point of lifespan there is an excess of infections and what is the temporal relationship of these infections to possible emerging autoantibody seroconversion and onset of T1D. For example, an excess of enterovirus infections has been found prior to autoantibody seroconversion by detecting enterovirus RNA in serial serum and stool samples collected from prospectively followed children in the Finnish DIPP study (22,112). These infections thus precede the activation of the process leading to T1D. However, prospective studies cannot provide information about the spread of the virus to different organs, and its possible persistence in the pancreas. Thus, information from tissue studies and clinical cohorts supplement each other and their combination offer an ideal approach to understand the pathogenesis of T1D.

6.7 Enterovirus persistence

Enterovirus persistence in the pancreas is currently one of the key hypothesis to explain the development of virus-induced T1D. Enteroviruses are considered cytolytic viruses but they are also able to establish persistent infections *in vitro* and *in vivo* (210,211). Persistence of a virus may lead to an inflammatory process, which in turn can contribute to the induction of autoimmune responses (212). Some enteroviruses may persist in the central nervous system (CNS) and, thus, avoid clearance by the host (213). Enterovirus persistence in the pancreas has been suggested to play a role in the process leading to T1D (212,214,215). Possible persistence would fit with the high proportion of T1D patients with enterovirus protein in beta cells and low titer of the virus that suggest low-grade rather than acute infection (130). In addition, there is no major lytic damage in the pancreata where enterovirus has been detected (130,131,140).

Additionally, it has been proposed that CV-B can persist in the host or host tissue in a terminally deleted form in which a deletion occurs in the 5'UTR of the viral genome. This leads to a reduced cytopathic effect and reduced viral titers, an observation that was first described by Kim et al. in 2008 and later also by Tracy et al. in 2015 using murine model (177,178). The low-level replication of persisting virus would make it challenging to detect the virus in tissue samples.

6.8 Future prospects

Large international studies like nPOD, PEVNET and TEDDY and the Finnish DIPP study are vital for the future progress in this field. These studies have already built a basis for the concept of intervention studies targeting enteroviruses, such as primary prevention by vaccination or secondary prevention by antiviral treatments. These intervention studies could provide information about the possible causal nature of the enterovirus-T1D association. For example, a finding showing that an enterovirus vaccine prevents infections by T1D-associated enterovirus types and decreases the occurrence of T1D, would strongly support causality. The first steps have already been taken in the vaccine development. Experimental CV-B1 vaccines have been highly immunogenic and able to protect mice against a CV-B1 infection and against CV-B1-induced diabetes (216). In addition, a polyvalent CV-B vaccine showed a similar result (217). These experimental vaccines have not accelerated the development of T1D in NOD mice. Moreover, the clinical development of a polyvalent CV-B vaccine has started aiming at first human trials in 2020. The ultimate

goal is to study whether this vaccine can reduce the incidence of T1D in a phase III trial. The fact that poliovirus vaccines have been safe and effective offers a model that helps the development process. Enteroviruses are also associated with many other diseases, and enterovirus vaccine could offer other advantages beyond T1D as well. Antiviral drugs given to patients with T1D-related autoantibodies could eradicate the possible chronic infection from the pancreatic islets of these patients, possibly improving the function of beta cells and, in the best-case scenario, prevent the disease. (218) The first trial to test the effect of antiviral drug treatment (combination of pleconaril and ribavirin) on the beta cell function among newly diagnosed T1D patients has recently started in Norway (Diabetes Virus Detection and Intervention Trial, DiViDinT).

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9 ORIGINAL COMMUNICATIONS

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Application of bioinformatics in probe design enables detection of enteroviruses on different taxonomic levels by advanced *in situ* hybridization technology

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ABSTRACT

Background: Enteroviral infections are common, affecting humans across all age groups. RT-PCR is widely used to detect these viruses in clinical samples. However, there is a need for sensitive and specific in situ detection methods for formalin-fixed tissues, allowing for the anatomical localization of the virus and identification of its serotype.
Objectives: The aim was to design novel enterovirus probes, assess the impact of probe design for the detection and optimize the new single molecule *in situ* hybridization technology for the detection of enteroviruses in formalin-fixed paraffin-embedded samples.
Study design: Four enterovirus RNA-targeted oligonucleotide RNA probes – two probes for wide range enterovirus detection and two for serotype-targeted detection of Coxsackievirus B1 (CVB1) – were designed and validated for the commercially available QuantiGene ViewRNA *in situ* hybridization method. The probe specificities were tested using a panel of cell lines infected with different enterovirus serotypes and CVB infected mouse pancreata.
Results: The two widely reactive probe sets recognized 19 and 20 of the 20 enterovirus serotypes tested, as well as 27 and 31 of the 31 CVB1 strains tested. The two CVB1 specific probe sets detected 30 and 14 of the 31 CVB1 strains, with only minor cross-reactivity to other serotypes. Similar results were observed in stained tissues from CVB –infected mice.
Conclusions: These novel in-house designed probe sets enable the detection of enteroviruses from formalin-fixed tissue samples. Optimization of probe sequences makes it possible to tailor the assay for the detection of enteroviruses on the serotype or species level.

1. Background

Enteroviruses[1] (EVs) often cause mild, common flu-like symptoms, but they can also be responsible for more severe conditions, such as myocarditis, central nervous system infections and poliomyelitis [1]. Currently, the over 110 human EV serotypes are divided into four species termed Enterovirus A–D. Increasing evidence suggests that these different EV serotypes play a role in the

[1] EV, enterovirus.

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pathogenesis of acute [2,3] and chronic diseases; e.g., EVs of species B have been linked to the pathogenesis of myocarditis [4,5] and type 1 diabetes[2] (T1D) [6,7]. Current evidence also suggests that EVs can establish a slowly replicating persistent infection in the pancreas [8,9]. Furthermore, recent epidemiological studies have suggested that some group B coxsackieviruses[3] (CVBs), belonging to the EV B species, are linked to the initiation of pancreatic beta-cell autoimmunity and development of T1D [10,11]. The causal association between EVs and T1D however, remains uncertain and further studies are needed to verify this question.

The interest in the direct detection of EVs in their target organs has increased in the last few years. These viruses have caused severe epidemics (e.g., those with CVB1, EV68 and EV71) and place an increasing burden on society. While RT-PCR allows for the sensitive detection of EVs in clinical samples, it does not work optimally in formalin-fixed paraffin embedded[4] (FFPE) tissue samples and can also not be used for the localization of the virus in specific cells. In fact, a method capable of detecting a broad range of EVs and distinguishing individual serotypes directly in FFPE tissue samples has not been reported. Methods suited for EV detection in FFPE samples would offer important advantages: (1) most tissue sample collections are based on FFPE samples (2) such samples preserve the histological structures. Thus far, immunohistochemistry-based studies on EVs have mostly been based on a single commercially available antibody clone 5D8/1 (Dako, Glostrup, Denmark) that recognizes viral capsid protein VP1[5] [12]. This method has provoked discussions with regards to its sensitivity and specificity in recent publications [13–16]. Combining viral protein findings with standardized and reliable *in situ* hybridization[6] (ISH) mRNA results would strengthen the reliability of viral detection. Further, as EV serotypes have different pathogenic features, there is a clear need for a standardized method capable of distinguishing these viruses; not only species-specifically but also group- or serotype specifically.

The challenges posed with detecting viruses using ISH, especially RNA viruses such as EVs, include the lack of a common nucleotide sequence in all viruses, high sequence variation and genomic recombination. Nevertheless, these characteristics also enable both narrow and broad genotypic detection design for various EVs. Firstly, different gene regions common in varying taxonomic levels can be utilized in a specific probe design depending on how broad the genotype detection is wanted. Secondly, loci of the target probe sequences can be selected from genomic regions with either high or low variation and thirdly, the design of the probe sequence can be based on either a single genotype sequence or a consensus sequence of various enteroviral genomes.

2. Objectives

The aim of this study was to broaden the methods available for EV detection in tissue samples. We developed a novel specific ISH method for selected EV species, as well as serotype-specific assays, using a commercial and standardized non-radioactive Affymetrix QuantiGene ViewRNA system for FFPE cell and tissue samples.

3. Study design

3.1. Enterovirus-infected cell culture samples

Green monkey kidney cells (GMK and Vero), carcinomic human cervix epithelial cells (HeLa), carcinomic human alveolar basal epithelial (A549) cells and human rhabdomyosarcoma muscle cells (RD) were grown in a monolayer in complete media (Supplementary, Table 1) with 5% FBS. The cell lines were infected with selected viruses (Supplementary, Table 2 and 3) in their individual T75 flask, until 50% cytopathic effect was reached, then harvested by scraping and fixed in 4% formaldehyde in PBS for 24–72 h prior to dehydration and paraffin embedding.

3.2. Animal husbandry and in vivo CVB infections in mice

Non-obese diabetic[7] (NOD) and C57BL/6J Rag$^{-/-}$ (B6 Rag-/-) mice were bred and housed in a specific-pathogen-free environment at Karolinska Institutet, Stockholm, Sweden. All mice were infected through the *intra peritoneal* (i.p.) route with different serotypes and doses of CVBs diluted in 200 μl RPMI (Supplementary, Table 4). Plain RPMI was used for mock-infected controls. Systemic infection was confirmed in blood by RT-PCR or plaque assay on day 3–5 post infection. Organs were harvested between day 3 and 7 post infection and fixed in 4% formaldehyde in PBS for 24 h prior to dehydration and paraffin embedding. All animal experiments were approved by a local ethics committee and conducted in accordance with the NIH principles of laboratory animal care and the Swedish law.

3.3. Probe set design

Characteristics of the EV genome were utilized in the optimization of the probe specificity. Two different probe sets were designed for individual EV serotype, namely probes CVB1 and CVB1Sub and two for species-specific detection, namely probes EV AB and EV B targeting mainly EV species A and B and EV B, respectively. The design was based either on a sequence of one viral strain (probes CVB1Sub and EV B) or on a consensus sequence of multiple viruses (probes CVB1 and EV AB). The CVB1Sub probe was designed based on a sequence in GenBank under the accession no. EU147493.1 and that of probe EV B on the accession no. KJ849619. For the probes where the design is based on a consensus sequence, all of the available full-length EV sequences in GenBank were used (28 sequences for CVB1 probe and 128 sequences for EV AB probe). In addition for CVB1 probe, the P1 region of 27 wild type CVB1 strains circulating in Europe, South- and North-America were sequenced. All these sequences were aligned using the Clustal X program. Analogous sequences were deleted to avoid the possible over impact of multiple similar sequences on the consensus sequence of the aligned CVB1 strains. The consensus sequence was extracted and used as a template for probe design.

The sequence of P1 region of the EV genome links to viral serotype, hence this locus was used solely as the serotype-specific probe targets (probes CVB1 and CVB1Sub). The 5' untranslated region[8] (UTR) is common between A and B species and similarly between C and D species, whereas P2 and P3 regions are relatively conserved within all EV species. To target probes at species level (probes EV AB and EV B) and to maximize the sensitivity, the conserved regions of 5′UTR, and, conserved loci from the non-structural P2 and P3 regions were used (Fig. 1, Table 1). Oligonucleotide

[2] T1D, type 1 diabetes.
[3] CVB, group B coxsackievirus.
[4] FFPE, formalin-fixed paraffin-embedded.
[5] VP, viral protein.
[6] ISH, in situ hybridization.
[7] NOD, non-obese diabetic.
[8] UTR, untranslated region.

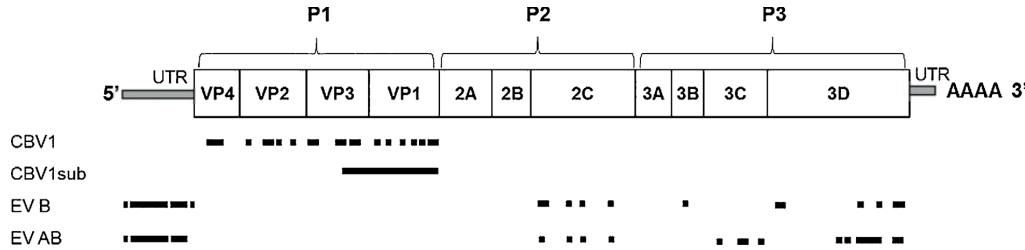

Fig. 1. Schematic presentation of the EV genomic structure. The positions of probes in the EV genome are marked with black lines.

Table 1
Description of the enterovirus probe sets.

Probe name	Target region length	Main target	Viral sequences used for probe design
EV AB	1359	Species A, B	Consensus sequences from - P2 and P3 regions of EV A - 5′UTR, P2, P3 regions of EV B
EV B	1063	Species B	GenBank accession no KJ849619 - 5′UTR, P2, P3 regions
CVB1	908	CVB1 strains	Consensus sequence of CVB1 strains from P1 region
CVB1Sub	960	Genetically close strainsof CVB1 EU147493	GenBank accession no EU147493 from P1 region

fragments from 33 to 51 nucleotides[9] (nt) long were selected (Supplementary, Table 5). Depending on the probe, these probe set pair fragments formed, as a whole, a target sequence with the length of 908 to 1359 nt (Table 1).

3.4. Quantigene ViewRNA in situ hybridization

The Quantigene ViewRNA ISH system is based on a similar probe set design and the branched DNA signal amplification technology, as the previously described RNAscope ISH [17], providing robust *in situ* detection of target mRNA from FFPE sections with single-copy sensitivity. The assay itself can be divided into four primary steps: sample preparation, target hybridization, signal amplification and detection with brightfield and/or fluorescent microscopy.

Viral RNA was stained and visualized in 5-μm paraffin-embedded sections from acutely enterovirus-infected cell culture samples and CVB-infected mouse pancreas according to the manufacturer's instructions. Briefly, FFPE sections were rehydrated, pretreated by 5 min boiling and incubated with Proteinase K (5 μg/ml in PBS) for 10 min. They were subsequently incubated with a viewRNA probe set designed against the mRNA of CVB1, or against the mRNA covering the conserved regions of Enterovirus B species (i.e., EV B probe set) or against the mRNA of the conserved regions from EV A&B species (i.e., EV AB probe set). After incubation the signal was amplified with alkaline phosphatase[10] (AP) –labeled probes and visualized under both brightfield and fluorescent light with objectives UplanFL 10X/0.30, UplanFL 20X/0.50, and UplanFL 40X/0.75, and an Olympus BX60 fluorescent microscope (Olympus, Melville, CA, USA).

3.5. Immunostaining with clone 5D8/1

Immunostaining with clone 5D8/1 (Dako, Glostrup, Denmark) to detect enterovirus capsid protein 1 (VP1) in cells was performed as previously described [18]. For murine tissue, FFPE pancreatic sections were cut into 5-μm sections and stained as previously described [19], with the following modifications; antigen retrieval was performed in 10 mM Tris and 1 mM EDTA, pH9, and sections were stained using a biotinylated clone 5D8/1 antibody (biotinylated by Capra Science, Ängelholm, Sweden).

All the stained slides were further analyzed using scanned slide images. The slides were scanned with a fully automated Objective Imaging Surveyor virtual slide scanner. Digitization was performed at a resolution of 0.4 microns per pixel (using 20X Plan Apochromatic microscope objective). Image data was converted to JPEG2000 format as previously described [20].

4. Results

The ability of the newly designed probe sets to bind their target mRNAs was examined using infected and non-infected cell lines, including a variety of different EV serotypes and strains (Supplementary, Table 2 and 3) and pancreas samples collected from mice infected with CVB serotypes 1, 3, 4, 5 and 6. The results are described in a table format valuing the binding to the mRNA target as follows: – = no binding; + = weak positive; ++ = positive; +++ = strong positive (Tables 2–4).

4.1. Detection of enteroviruses in infected cells

The EV AB probe recognized all tested viruses of EV species A and B. The recognition spectrum of EV B probe was similar to that of EV AB probe, with the exception of poliovirus 3[11] (PV3) which belongs to EV species C; however, the overall intensity of the staining of different serotypes was stronger with the EV AB probe (Fig. 2.). The CVB1 probe stained all but one CVB1 strains strongly, but also reacted weakly with some other CVB serotypes. The CVB1Sub probe gave a more narrow recognition spectrum among the tested CVB1 strains and it did not react with any other serotype. Every probe gave a negative result in the mock-infected cells as well as in the human parechovirus1[12] (HPeV1) and Adenovirus C infected cells (Tables 2–4).

[9] nt, nucleotide.
[10] AP, alkaline phosphatase.
[11] PV3, poliovirus 3.
[12] HPeV1, human parechovirus 1.

Table 2
Probe sets' recognition spectrum of various EV serotypes and control viruses. Binding of monoclonal antibody against EV VP1 is shown for comparison.

Virus		Probe sets				Anti-VP1 antibody
Species	Serotype	EV AB	EV B	CVB1	CVB1Sub	Clone 5D8/1
EV B	CVB1	+++	+++	+++	+++	+++
EV B	CVB2	+++	++	–	–	+++
EV B	CVB3	+++	++	–	–	+++
EV B	CVB4	+++	++	+	–	+++
EV B	CVB5	+++	+++	–	–	+++
EV B	CVB6	+++	++	–	–	++
EV B	Echo3	+++	++	–	–	+
EV B	Echo4	+++	+++	–	–	+
EV B	Echo6	+++	+++	+	–	+++
EV B	Echo9	+++	++	–	–	++
EV B	Echo11	+++	++	+	–	+
EV B	Echo30	+++	++	–	–	+++
EV B	CVA9	+++	++	–	–	+++
EV A	CVA2	++	+	–	–	+
EV A	CVA4	++	++	–	–	+
EV A	CVA5	+	+	–	–	++
EV A	CVA6	++	+	–	–	+
EV A	CVA10	+++	++	–	–	+
EV A	CVA16	+++	++	–	–	+
EV A	EV71	–	–	–	–	–
EV C	PV3	++	–	–	–	++
Adeno	VR846	–	–	–	–	–
HPeV	HPeV1	–	–	–	–	–

4.2. Detection of group B Coxsackieviruses in infected mice

The probes' ability to recognize their targets in pancreas samples from CVB infected mice was also tested. Every probe gave a positive signal when detecting their matching targets. The recognition pattern followed that seen in infected cell culture samples (Fig. 3). Some cross-recognition was observed with CVB1 probe, which also bound to CVB5 and weakly to CVB3 (Table 4).

5. Discussion

The results of the present study demonstrate that new single molecule ISH techniques can be used to detect EVs in FFPE samples. This technology also offers certain advantages over traditional ISH methods, as it is based on non-radioactive probes and as the flexible probe design enables the detection of either a wide range of different virus types or the identification of the exact serotype of

Table 3
Probe sets' recognition spectrum of different CVB1 strains. Binding of monoclonal antibody against EV VP1 is shown for comparison.

CVB1 Strain			Probe sets				Anti-VP1 antibody
ID	Place	Year	EV AB	EV B	CVB1	CVB1 Sub	Clone 5D8/1
PB-10787	GA, USA	1970	+++	–	+	+++	+++
PB-10789	ME, USA	1972	++	+	–	++	++
PB-10790	TN, USA	1973	++	++	++	+++	+++
PB-10791	NH, USA	1977	++	+	+	+	++
PB-10792	ME, USA	1979	++	+	++	++	++
PB-10794	SD, USA	1981	++	++	+	–	++
PB-10796	Argentina	1983	+++	+	+	Not analyzed	++
PB-10798	GA, USA	1985	+++	++	+++	+	+++
PB-10799	Honduras	1987	+++	++	++	+++	+++
PB-10800	TN, USA	1991	+++	++	+++	++	+++
PB-10802	Argentina	1998	+++	++	+++	–	+++
PB-10803	Chile	1998	+++	+++	+	–	+++
PB-10788	ME, USA	1971	+++	++	++	++	+++
PB-10793	RI, USA	1980	+++	++	+++	+	+++
PB-10795	GA, USA	1982	+++	++	++	+	+++
PB-10797	CT, USA	1984	+++	++	+++	–	+++
PB-10801	South Africa	1992	+++	++	++	–	+++
PB-5CWCS	COL, USA	2007	+++	++	+++	–	+++
PB-59XGU	GEO, USA	2007	+++	+++	++	–	+++
PB-5HTTV	GEO, USA	2008	++	+	++	–	++
PB-5HJXX	Germany	2008	+++	+	+++	–	+++
PB-59MG4	WAS, USA	2008	+++	+++	+++	–	+++
PB-5FAMZ	WAS, USA	2007	++	++	+++	–	+++
PB-59ZJJ	GEO, USA	2008	+++	++	+++	–	+++
ATCC			+++	+	++	++	+++
PB-CVB1V200	No data	No data	+++	–	++	+++	+++
CF217010_FRA08	France	2008	++	++	+++	–	++
CF1650_FRA02	France	2002	++	+	+++	–	+++
CF741_FRA93	France	1993	+	+	++	–	+
CF1887_FRA00	France	2000	+++	–	+++	–	+++
CF168014_FRA06	France	2006	+++	–	++	–	+++

Table 4
Probe sets' recognition spectrum of CVB serotypes 1,3,4,5 and 6 infected mouse pancreas. Binding of monoclonal antibody against EV VP1 is shown for comparison.

Virus		Mouse	Probe sets				Anti-EV VP1 antibody
Serotype	Strain		EV AB	EV B	CVB1	CVB1Sub	Clone 5D8/1
CVB1	V200	NOD	++	++	++	+++	++
CVB1	PB-10796	NOD	++	++	+	++	++
CVB1	PB-10802	NOD	++	++	+++	+	+++
CVB3	Nancy	B6Rag-/-	+++	+++	+	–	+++
CVB4	E2	NOD	++	++	–	–	++
CVB5	ATCC	NOD	++	+	++	–	+++
CVB6	ATCC	NOD	++	+	–	–	–

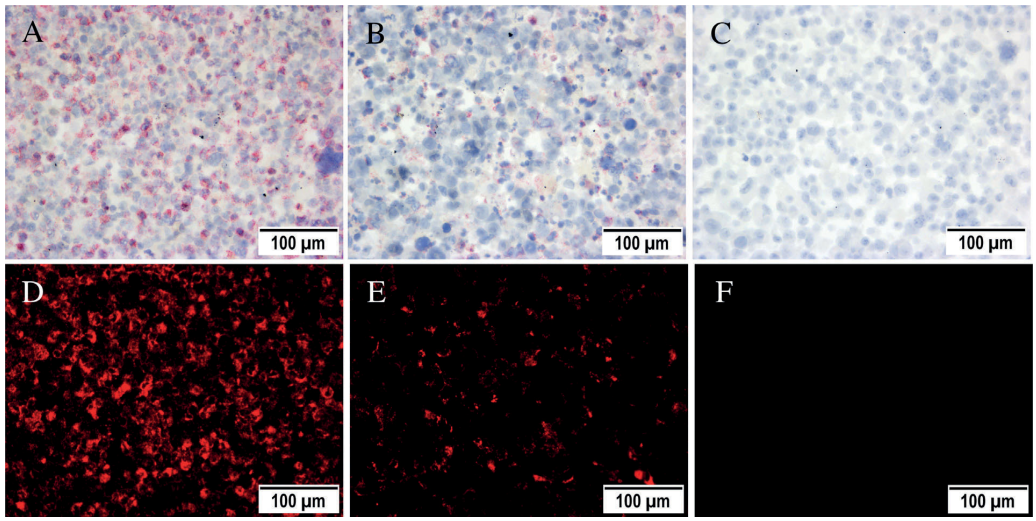

Fig. 2. ISH staining in CAV16 infected RD cells. (A) EV AB in infected cells, (B) EV B in infected cells, and (C) EV AB in non-infected cells, red color indicating enteroviral presence. Panels D, E and F represent the corresponding fluorescent images of A, B and C, respectively. Original magnification × 20. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

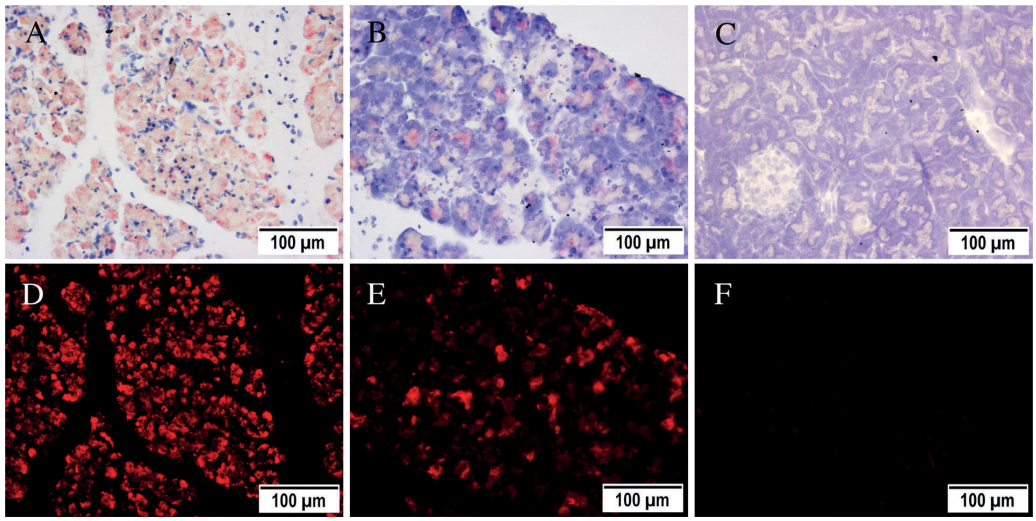

Fig. 3. ISH staining in infected murine pancreas. CVB3 infected (A), CVB5 infected (B), and non-infected (C) pancreas. Red color indicates enteroviral presence. Panels D, E and F represent the corresponding fluorescent images of A, B and C. Original magnification × 20.

virus(es) present in tissue samples, making this technique optimal for research and diagnostic purposes.

Four different probe sets were designed, two for wide range EV recognition and two for specific EV serotype recognition. The most conventional approach was applied to the design of the CVB1Sub probe set by choosing a continuous sequence of 960 nt covering parts of the VP3 and VP1 regions. Consequently, this probe only detected CVB1 strains. A different approach was selected for the CVB1 probe set, where conserved parts from the P1 region of a consensus sequence found in many CVB1 strains was used as a basis for the design. Accordingly, the CVB1 probe detected all but one of 31 CVB1 strains tested. Additionally, sequences from newer circulating strains were prioritized in the CVB1 probe sequence design, which is also reflected in the results by overall weaker recognition among the older CVB1 isolates (PB-10787 to PB-10796). Despite the wide serotype-specific recognition, minor cross-detection of a small number of other EV serotypes was also observed, particularly CVB5 (ATCC). In spite of the monotypic recognition of CVB1Sub probe, as expected, its recognition spectrum was significantly narrower compared to the CVB1 probe. Traditionally, ISH probes are produced by digesting full-length genomes or long consecutive regions of one EV strain. Our results show that this kind of probe (e.g., CVB1Sub) is more likely to detect a narrow spectrum of genetically similar viruses, therefore making it a non-optimal screening probe for clinical samples that may be infected with unknown EV types.

Both of the probes that were originally designed to cover many EV serotypes recognized a majority of the EV serotypes tested. Although the original aim behind the design was similar for both EV AB and EV B probes, the former had a better recognition profile and stronger intensity of the staining. The main difference between these probes lies in the design basics; the design of EV B probe was based on conserved sequence regions of a single CVB1 strain, whereas consensus sequences of multiple viruses were used for the optimal probe sequence design of EV AB probe. Also, the overall target length for the latter was approximately 25% longer, and included more target regions for EV species A, which is reflected in the results by a slightly better recognition of group A Coxsackieviruses[13] (CVAs).

When comparing the recognition pattern of EV AB probe to the anti-VP1 antibody clone 5D8/1, it was observed that both bind very well to CVBs and echoviruses. On the other hand, the relative immunoreactivity of clone 5D8/1 to CVAs is poorer than the target binding ability of EV AB probe to CVAs (Table 2). Both detected CVB1 strains equally well (Table 3) and similar results were seen in infected mice (Table 4). Hence, we conclude that this probe is a valuable tool for EV detection. However, whether the sensitivity of EV AB ISH matches that of clone 5D8/1 immunohistochemistry remains to be confirmed.

In addition to the aspect of sensitivity, detection of viral mRNA in itself brings challenges, as the viral presence in target tissues is likely to be very low. Also, the multiple RNases present in the tissues might have an effect on the results through weakening the signal by breaking down the viral RNA. Moreover, mRNA detection in FFPE tissues is challenging due to extensive molecular crosslinking that occurs upon formalin fixation. Regardless of these, we were able to establish this method on FFPE tissues. Yet, some probe sets might work even better in frozen sections, as has been previously reported with different antibodies [21].

In conclusion, our results show that the novel probes, together with the commercial ViewRNA ISH system, enabled the localization of mRNA in cell culture and animal models and therefore provides a potential method to study the course of acute viral infections in animals and humans. Based on these findings this method can be used in both the broad screening for EVs and for the identification of the exact serotype of the infecting virus. Also, this strategy is probably applicable for other viruses when identification of the serotype is required in FFPE samples.

[13] CVA, group A Coxsackievirus.

Conflict of interest

None declared

Funding

None

Ethical approval

Animal experiments were approved by ethics committees of Karolinska Institutet (MFT) and conducted in accordance with the NIH Principles of Laboratory Animal Care and national laws in Sweden

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2015.06.085

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Relative sensitivity of immunohistochemistry, multiple reaction monitoring mass spectrometry, *in situ* hybridization and PCR to detect Coxsackievirus B1 in A549 cells

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ABSTRACT

Background: Enteroviruses (EVs) have been linked to the pathogenesis of several diseases and there is a collective need to develop improved methods for the detection of these viruses in tissue samples.
Objectives: This study evaluates the relative sensitivity of immunohistochemistry (IHC), proteomics, *in situ* hybridization (ISH) and RT-PCR to detect one common EV, Coxsackievirus B1 (CVB1), in acutely infected human A549 cells *in vitro*.
Study design: A549 cells were infected with CVB1 and diluted with uninfected A549 cells to produce a limited dilution series in which the proportion of infected cells ranged from 10^{-1} to 10^{-8}. Analyses were carried out by several laboratories using IHC with different anti-EV antibodies, ISH with both ViewRNA and RNAScope systems, liquid chromatography multiple reaction monitoring mass spectrometry (LC/MRM/MS/MS), and two modifications of RT-PCR.
Results: RT-PCR was the most sensitive method for EV detection yielding positive signals in the most diluted sample (10^{-8}). LC/MRM/MS/MS detected viral peptides at dilutions as high as 10^{-7}. The sensitivity of IHC depended on the antibody used, and the most sensitive antibody (Dako clone 5D8/1) detected virus proteins at a dilution of 10^{-6}, while ISH detected the virus at dilutions of 10^{-4}.
Conclusions: All methods were able to detect CVB1 in infected A549 cells. RT-PCR was most sensitive followed by LC/MRM/MS/MS and then IHC. The results from this *in vitro* survey suggest that all methods are suitable tools for EV detection but that their differential sensitivities need to be considered when interpreting the results from such studies.

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1386-6532/

1. Background

Enterovirus[1] (EV) infections are common in all age groups. They are usually asymptomatic or cause only mild respiratory symptoms, but can also lead to more severe illness including hand, foot and mouth disease, myocarditis, meningitis, encephalitis, pancreatitis, systemic infection in newborns and paralysis. EV infections may also play a role in the pathogenesis of chronic diseases such as dilated cardiomyopathy [1], chronic fatigue syndrome [2] and type 1 diabetes[2] (T1D) [3–5].

Laboratory diagnosis of EV infection is based on virus detection in stools, nasal/throat swabs or cerebrospinal fluid, as well as on EV-specific antibody responses in serum. However, studies evaluating the pathogenesis of EV infections and their possible role in chronic diseases (where levels of viral infection may be low but persistent) require additional technologies and there is an increasing need for direct virus detection in tissue samples. Traditionally, EVs are detected in tissue samples either by RT-PCR or immunohistochemistry[3] (IHC). In addition to these, the new single molecule hybridization [6,7] as well as mass spectrometry/proteomics technologies offer new opportunities for viral detection. However, there are no previous reports in which the performance of these various technologies has been evaluated in relation to one another.

2. Objectives

The aim of the study was to evaluate the relative sensitivities of proteomics, ISH, IHC and RT-PCR methods to detect one common EV, Coxsackievirus B1[4] (CVB1), using human A549 cells diluted to contain differing ratios of uninfected to *in vitro* EV-infected cells.

3. Study design

3.1. Preparation of EV-infected cell arrays

Human A549 alveolar basal epithelial cells were grown in monolayers in Nutrient Mixture F-12Ham, N 6658 (Sigma–Aldrich®) medium in T175 bottles and infected with CVB1, ATCC strain (10–15 MOI). The infection was stopped at four different time points (1 h, 2 h, 4 h, and 6 h post infection) to obtain a series of infected cells representing different stages of viral replication cycle. The cells from different time points were mechanically detached, pooled and washed with the growth medium. The cells were then immediately diluted with uninfected A549 cells to produce a dilution series ranging from 10^{-1} to 10^{-8}, as well as an undiluted sample (positive control) and a sample of uninfected A549 cells (negative control). Each dilution aliquot was further divided into ten sub-aliquots, each containing about 1 million cells. These sub-aliquots were fixed or stored in an optimal way for each of the different methodologies employed. Some of the aliquots were fixed in 10% neutral-buffered formalin for 24 h and paraffin-embedded using standard procedures for IHC and ISH analyses. The rest were quickly frozen in liquid nitrogen and stored at −70 °C for RT-PCR and proteomics analyses. From the individual formalin-fixed paraffin-embedded[5] (FFPE) samples, a cell microarray was created using TMA Master (3D Histech Kft, Hungary) and 5 μm-thick sections were cut for histological stainings.

3.2. RT-PCR

RT-PCR was performed in two different laboratories (Tampere and Uppsala), each analyzing similar aliquots of the dilution series. In Tampere, RNA was extracted from 140 μl cell sample using the Viral RNA Kit (Qiagen, Hilden, Germany), and real-time RT-PCR was performed as previously described [8]. In Uppsala, viral RNA was extracted from 100 μl using RNeasy Mini kit (Qiagen). 50 ng total RNA/sample were primed with virus specific primers and reverse transcribed to cDNA with SuperScriptII™ RT (Invitrogen) according to the manufacturer's instructions. A semi-nested EV PCR was performed as described previously [3].

3.3. Proteomics

The dilution series samples were solubilized using 50% Trifluoroethanol in 50 mM ammonium bicarbonate as previously described [9]. Protein concentration was determined by bicinchoninic acid[6] (BCA) assay (Pierce, Rockford, Ill.). Concentration normalized samples from each of the dilution steps were reduced and alkylated as previously described [10]. Proteins were digested with trypsin at a ratio of 1:50 at 37 °C for 18 h to generate peptides. The peptides were purified using C18 columns, eluted using 80% acetonitrile in 0.1% formic acid and dried in a SpeedVac. Peptides were reconstituted in 0.1% formic acid prior to liquid chromatography multiple reaction monitoring mass spectrometry[7] (LC/MRM/MS/MS). LC/MRM/MS/MS on a triple quadrupole (QqQ) mass spectrometer[8] provides superior rapid, sensitive, and specific identification and quantitation of targeted compounds in highly complex samples [11,12].

LC/MRM/MS analysis of the tryptic peptides from the ten A549 dilution series cell samples was performed on a 4000 QTRAP® mass spectrometer coupled to a Tempo NanoLC system (ABSciex, Foster City, CA) [10]. Skyline was used to generate and optimize tryptic peptides and tandem MS/MS fragmentation data for developing MRM transitions pairs for CVB1 peptides [13]. A CVB1 2C protein peptide SVATNLIGR was selected for subsequent analysis and quantitation based on its abundance and high signal intensities for both the precursor ion (Q1 m/z) and fragment ions (Q3 m/z) and absence of signals in non-infected A549 cells. MRM Pilot ™ software (ABSciex) was used to optimize the assay conditions for the SVATNLIGR peptide with the following transition pairs of 465.7720/572.3515 and 465.7720/744.4363. The Q1 m/z (465.7720) for the MH^{2+} peptide parent mass and the Q3 m/zs correspond to y5 (572.3515) and y7 (744.4363) fragment ions. The final MRM assay conditions are detailed in Supplementary data.

The tryptic peptides corresponding to 1.6 μg of sample were injected and analyzed for each sample. The samples were sequentially analyzed starting with the non-infected samples and the low dilutions, to the non-diluted samples with multiple washing steps using blanks (buffer A) between each sample to avoid carry-over. Each sample was analyzed in duplicate experiments. Relative quantitation was achieved by comparing the area under the curve for the peptide transition pairs in the extracted ion chromatograms[9] (XIC) for each dilution step. The acquired data were processed and analyzed using Analyst 1.2 (ABSciex).

[1] EV, enterovirus.
[2] T1D, type 1 diabetes.
[3] IHC, immunohistochemistry.
[4] CVB, Coxsackievirus B.
[5] FFPE, formalin-fixed paraffin-embedded.
[6] BCA, bicinchoninic acid.
[7] LC/MRM/MS/MS, liquid chromatography multiple reaction monitoring mass spectrometry.
[8] QqQ, triple quadrupole mass spectrometer.
[9] XIC, extracted ion chromatogram.

3.4. Immunohistochemistry

IHC was performed in three laboratories (Tampere, Exeter and Uppsala). Primary analyses were done using a commercially available antibody raised against EV VP1[10] protein (clone 5D8/1; DAKO, Glostrup, Denmark). In Tampere and Exeter, IHC was performed as previously described [5,14,15]. In Uppsala, the sections were counterstained with haematoxylin (DAKO) before the addition of the primary antibody (diluted 1:2000) in Autostainer Link 48 (DAKO). Visualization was achieved with the DAKO Envision K8000. In addition, polyclonal antibodies produced in rabbits (see Supplementary) against each of the viral capsid proteins VP1, VP2, VP3 and VP4 of CVB4 Tuscany strain, were analyzed in the Tampere and Exeter laboratories. Antibodies from the first bleed (VP1A and VP3A) and from the last bleed (VP1B, VP2B, VP3B and VP4B) were used. In Tampere, staining was performed using the automated system and similar conditions to those for clone 5D8/1. In Exeter, following heat-induced epitope retrieval in 10 mM citrate, pH 6.0, the VP1-VP4 antibodies were incubated for 1 h at room temperature. The DAKO Envision Detection System was used for antigen detection as per the manufacturer's instructions and sections were counterstained with haematoxylin. The concentrations of CVB4 VP1-VP4 antibodies used in both laboratories are detailed in Supplementary Table 1.

3.5. In situ *hybridization*

ISH assays were performed in two laboratories (Tampere and Gainesville). In Tampere, the QuantiGene® ViewRNA (Affymetrix, Santa Clara, California, USA) was used with two different EV-specific probe sets (EV AB and CVB1), according to the manufacturer's instructions and as previously described [6]. In Gainesville, ISH was performed using the RNAscope 2.0 High Definition Assay (Advanced Cell Diagnostics, Hayward, California, USA) according to the manufacturer's instructions. Two EV-specific probes were tested to detect serotypes CVB1-6 and CVB3. Deparaffinized sections were hybridized to probes followed by amplification by serial application of amplifiers followed by peroxidase labels and detection with DAB.

4. Results

4.1. RT-PCR

Viral RNA was detected by RT-PCR in all samples although the semi-nested method was most sensitive. This yielded a positive signal from even the most dilute sample (10^{-8}) whereas the real-time RT-PCR method gave a positive signal in the second most dilute sample (10^{-7}). Ct values from real-time RT-PCR experiments with different dilutions of infected cells are shown in Supplementary Table 2.

4.2. Proteomics

MS-based targeted LC/MRM/MS/MS assay focused on the CVB1 2C protein peptide SVATNLIGR and the peptide signal was detectable at cell dilutions as high as 10^{-7}. Fig. 1shows the LC/MRM/MS/MS results for the relative abundance of the 2C protein peptide in undiluted, virus-infected cells. It also shows the detection of the MRM transition pairs signals, and the enhanced product ion scan[11] (EPI) showing the MS/MS fragmentation spectrum for the peptide.

[10] VP, viral protein.
[11] EPI, enhanced product ion scan.

Table 1
Comparison of the sensitivity of different methodologies to detect CVB1 in A549 cells.

Method	Sensitivity (dilution)
RT-PCR (frozen cells)	
Semi-nested (Uppsala)	10^{-8}
Real-time (Tampere)	10^{-7}
Proteomics (frozen cells)	
LC/MRM/MS/MS	10^{-7}
MRM	10^{-7}
IHC (FFPE cells)	
Anti-EV VP1: Clone 5D8/1	10^{-4}–10^{-6}
Anti-CVB4 VP1, –VP2, –VP3, –VP4	10^{-2}–10^{-4}
ISH (FFPE cells)	
Probes: EV AB[a], CVB1[b] (Affymetrix)	10^{-4}
Probe: CVB1-6, CVB3 (RNAscope)	10^{-4}

[a] Targets members of EV species A and B.
[b] Serotype-targeted probe.

Fig. 2 shows extracted ion chromatograms of the two transition pairs 465.7720/572.3515 (red) and 465.7720/744.4363 (blue) for the non-diluted, infected A549 cells (Panel A), the dilution series of the infected cells (Panels B–J) and the non-infected A549 cells (Panel K). The relative intensity and the accompanying signal for the MRM assay decreases from that of the peptide.

4.3. Immunohistochemistry

IHC also proved to be a sensitive method for detection of viral protein but was less sensitive than semi-nested RT-PCR, real-time RT-PCR and LC/MRM/MS/MS. IHC detected viral protein in virtually every cell in the undiluted sample, but the proportion of virus-positive cells decreased linearly as the dilution increased. Clone 5D8/1 was the most sensitive antibody tested detecting virus-positive cells at dilutions equal to or lower than 10^{-4} in Uppsala, 10^{-5} in Tampere and 10^{-6} in Exeter. At dilutions from 10^{-3} and beyond, the number of virus-positive cells was scarce, with only occasional cells stained positively (Fig. 3). In Tampere and Exeter, antibodies raised against CVB4 viral capsid proteins, stained efficiently the infected cells diluted over the range 10^{-2} to 10^{-4}. The intensity of the staining with these antibodies varied to some extent, with the VP1 (10^{-3}) and VP3 (10^{-4}) antibodies giving the highest sensitivities which were comparable in both Tampere and Exeter laboratories.

4.4. In situ *hybridization*

Both ISH methods (ViewRNA and RNAscope) demonstrated equal sensitivity, regardless of the probe used, detecting the virus at dilutions of 10^{-4} (Fig. 4). In the undiluted sample, almost all cells were EV-positive, and the number of positive cells decreased linearly as the ratio of CVB1-infected cells to uninfected cells was reduced.

Comparison of the sensitivity results between the methodologies is summarized in Table 1.

5. Discussion

The present study provides important information to guide the selection of assays capable of optimally detect EVs in infected cells. Although the conditions prevailing in mammalian cells infected *in vitro* do not completely resemble those in clinical tissue samples, the results provide a firm indication of the sensitivity and specificity of each method.

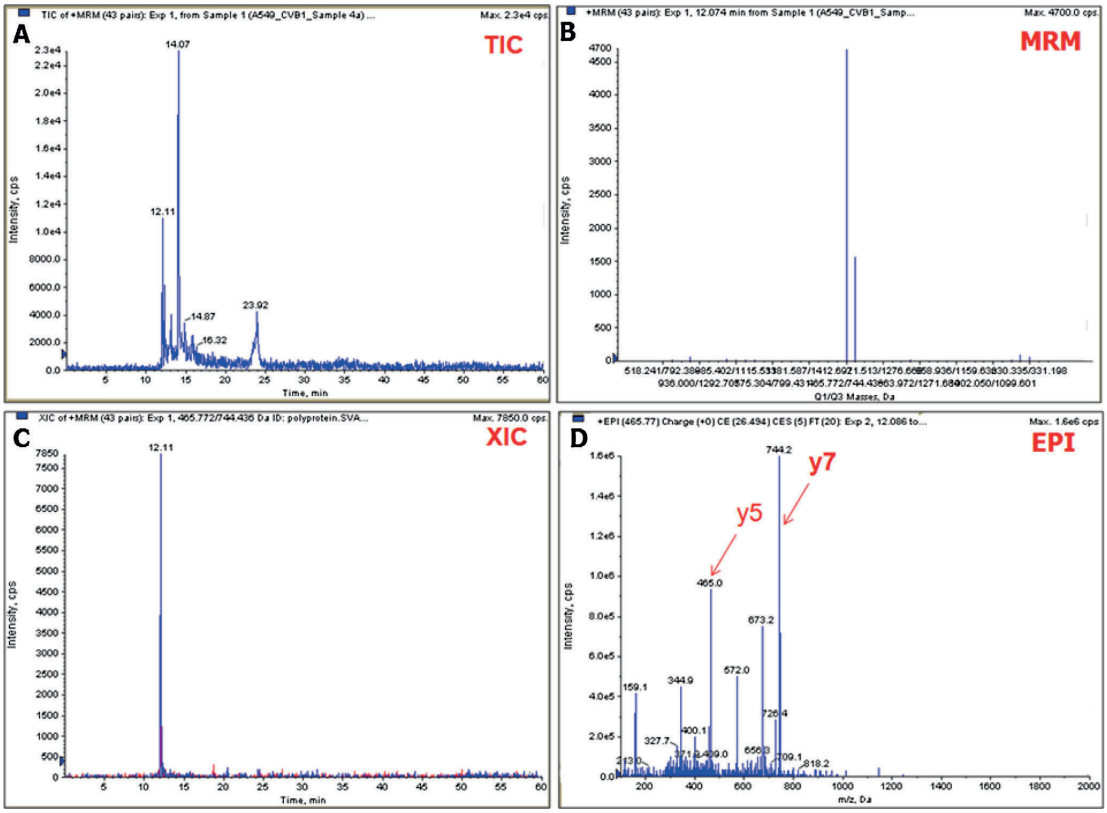

Fig. 1. Multiple reaction monitoring assay for CVB1 virus peptides. The total ion chromatogram and MRM peaks are shown in panels A and B. The extracted ion chromatogram (XIC) for the Protein 2C peptide is shown in panel C and the enhanced product ion scan (EPI) spectrum with the Q3 y5 and y7 fragment ions are shown in panel D. These two ions are the most intense in the tandem mass spectrum and their primary sequences correspond to the following c-terminus fragments of the peptide. y5 NLIGR (m/z = 572.3515) and y7 ATNLIGR (m/z = 744.4363).

All methods tested were able to detect CVB1 with good sensitivity. However, depending on the method used, the detection limit varied and RT-PCR was found to be the most sensitive one.

The new LC/MRM/MS/MS technology also demonstrated high sensitivity, while its sensitivity might be still further improved by use of higher capacity columns that allow the loading of larger amounts of peptides. This technology has the particular advantage that identification and validation experiments are performed at the same time; the overlapping extracted ion chromatograms for the MRM transition pairs provide confirmation that the signals are derived from the same peptide and the MS/MS spectrum data can be used for protein identification in database searches. These data highlight the potential utility of using modern sensitive MS approaches to identify viral sequences with a relatively high sensitivity, suggesting that its applicability for virus detection in human samples should be evaluated in detail. The differences in sensitivities observed among the laboratories using IHC approaches with the same antibody, and also the laboratories employing ISH, could be explained by the low number of virus-positive cells present in the more diluted samples. Once these dilutions are reached, the actual number of virus-positive cells is very low (1–2 cells per field); thus, positivity may vary from one section to another when cells are plated for analysis. Importantly, the different antisera tested exhibited broadly similar profiles among the different laboratories with the 5D8/1 clone consistently demonstrating the highest sensitivity. ISH sensitivity depends on a number of variables including the affinity with which the relevant probe sets bind to the CVB1 genome. Therefore, we cannot conclude that IHC and ISH data have yielded absolute sensitivities in each laboratory, but rather they provide an indication of the sensitivity range of each method.

Each of the tested methods clearly has its own advantages. The proteomics-based LC/MRM/MS/MS provides important molecular information about the detected viruses based on peptide sequences. We have also previously used mass spectrometry imaging[12] (MSI) to identify insulin and other proteins in pancreas tissue since the technology is useful for the identification and determination of the spatial distribution of molecules in tissues [16]. The preservation of tissue morphology is a clear advantage of IHC and ISH, thereby making it possible to localize the virus in individual cells. ISH appeared to be less sensitive than IHC, but this varied according to the type of antibodies used in IHC. The main advantage of RT-PCR is its high sensitivity and the possibility to derive sequence information from the viral genome.

This study also has certain limitations. Firstly, it was based on infected cells and not *ex-vivo* tissue samples, and therefore the relative sensitivity of each assay might be different when tissues are examined. Secondly, a single EV strain was used and, theoret-

[12] MSI, mass spectrometry imaging.

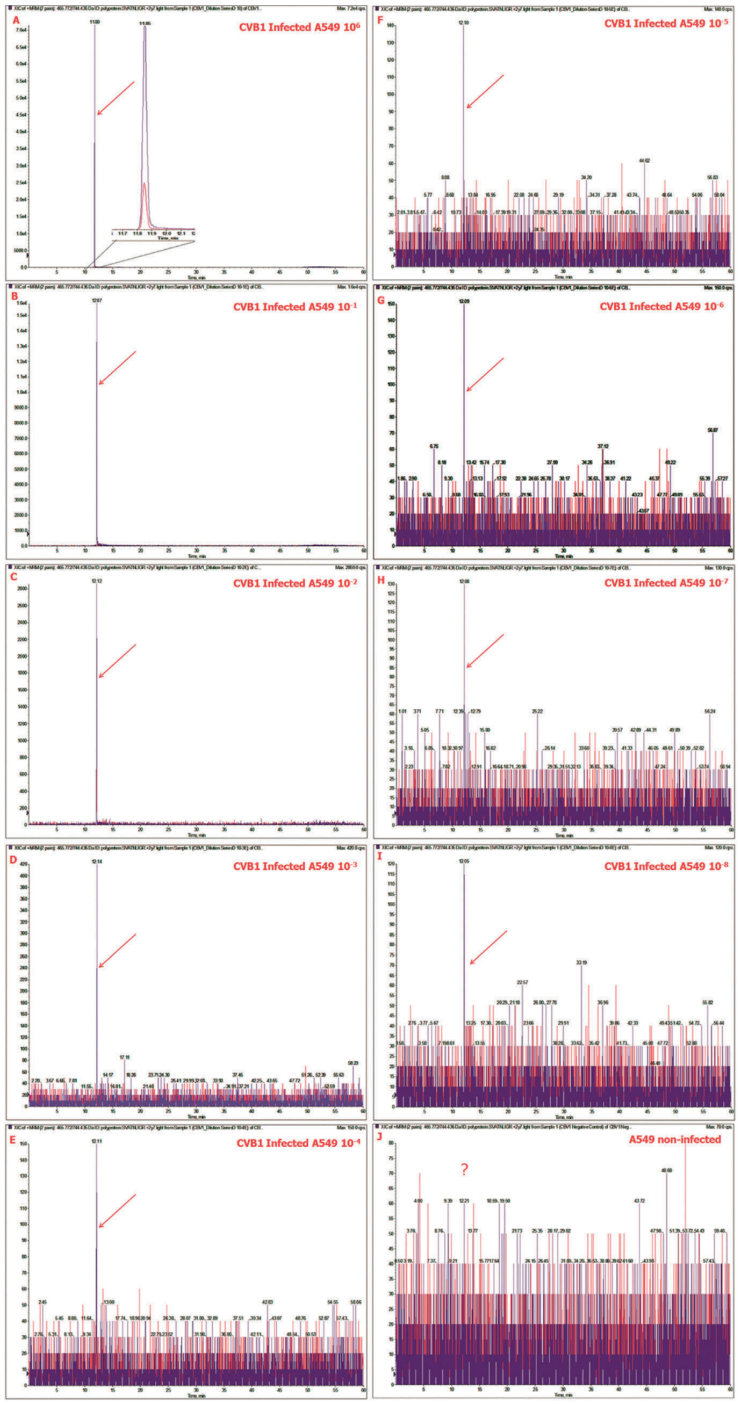

Fig. 2. MRM detection of CVB1 peptide in A549 cells LC/MRM/MS. Extracted ion chromatograms of the two transition pairs 465.7720/572.3515 (red) and 465.7720/744.4363 (purple) for the non-diluted CVB1 infected A549 cells panel (A), the dilution series of the infected cells (Panels B–I) and the non-infected A549 cells (Panel J). The MRM peaks in the samples are marked with a red arrow. Note the absence of signal (?) in Panel J. In panel A, the Zoom shows an expansion of the baseline to show the well resolved peaks of the extracted ion chromatograms of the two MRM transition pairs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

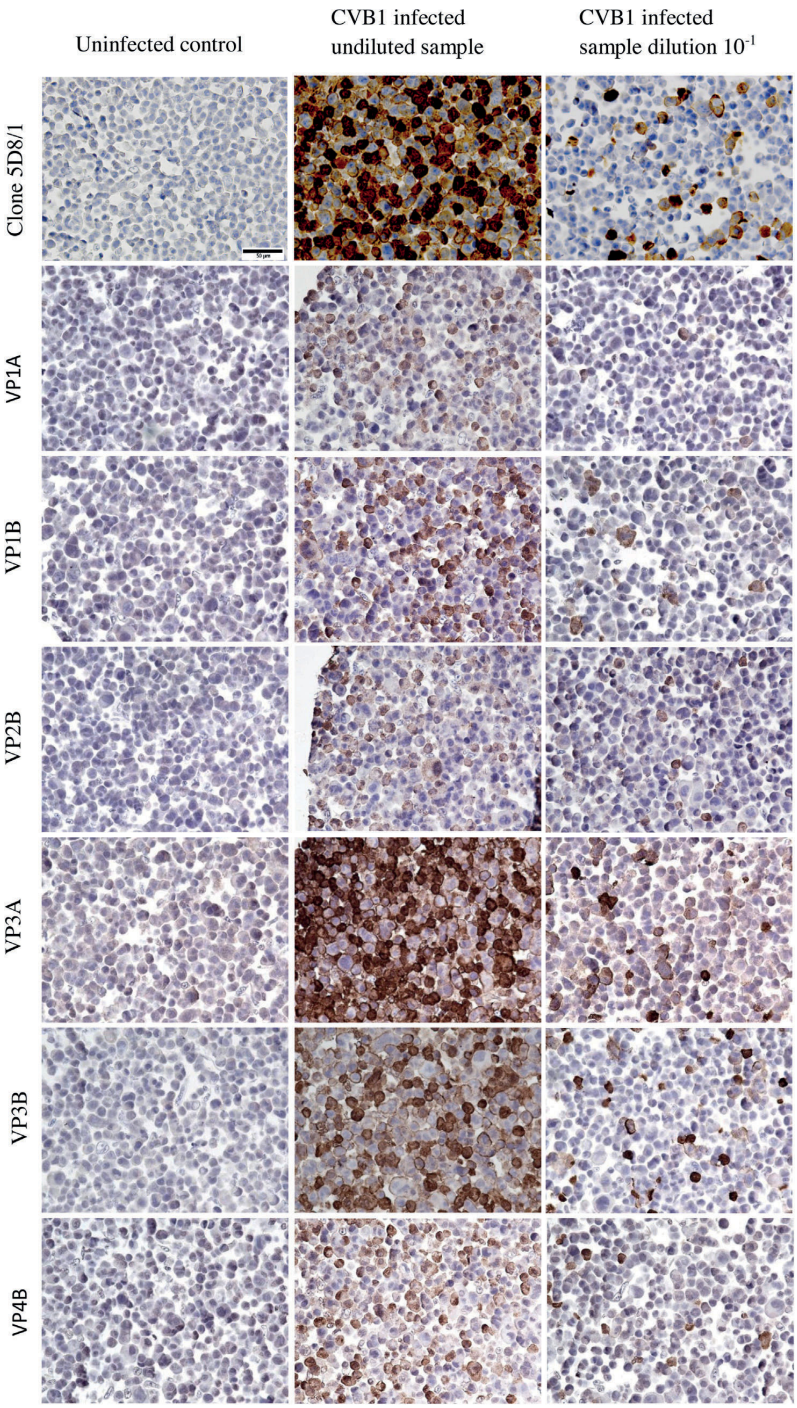

Fig. 3. Detection of CVB1 in infected A549 and uninfected A549 cells (FFPE) with different antibodies; commercial DAKO clone 5D8/1 and in-house anti-CVB4 antibodies VP1A, VP1B, VP2B, VP3A, VP3B and VP4B. Example micrographs of uninfected control, CVB1 infected undiluted sample and CVB1 infected dilution 10^{-1} are shown. 40× magnification. Scale bar = 50 μm.

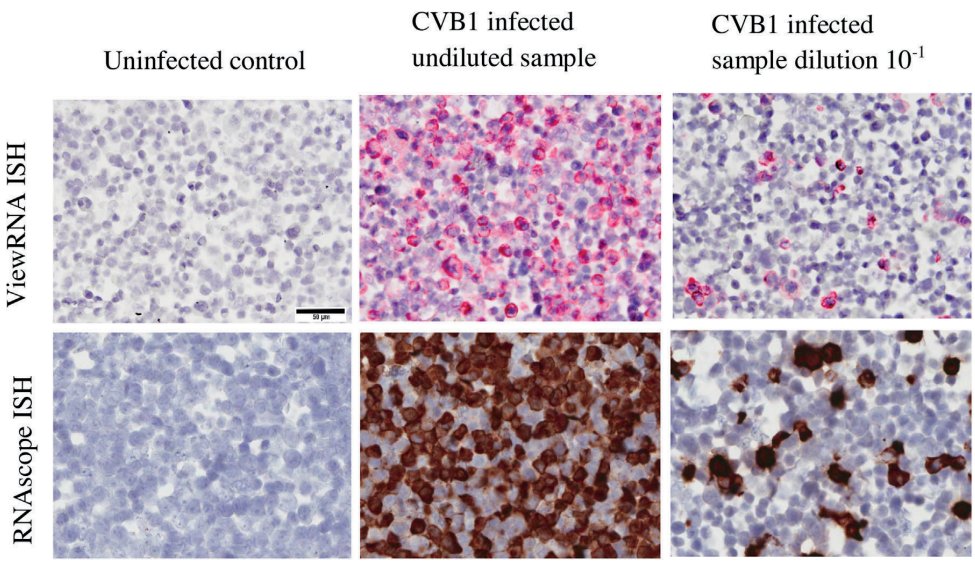

Fig. 4. Detection of CVB1 in infected A549 and in uninfected A549 cells (FFPE) using two different commercially available ISH (ViewRNA and RNAscope) methods. Example micrographs of uninfected control, CVB1 infected undiluted sample and CVB1 infected dilution 10^{-1} are shown. 40× magnification. Scale bar = 50 μm.

ically, the binding of antibodies, probes and primers to different EV strains may differ. However, the used antibodies bind to several different EV serotypes and strains, the used PCR primers amplify practically all EVs, the used ISH techniques allow specific probes to be designed, enabling the detection of the virus of interest [6], and proteomics analyses are not dependent on the EV type, suggesting that other EV strains should give comparable results. Third, it is difficult to exclude the possibility that in spite of the repeated washes of the infected cells during the preparation of infected cell arrays, remnants of extracellular viral peptides and RNA may have remained in the samples. This could have led to overestimation of the PCR and proteomics sensitivity, which can detect both intracellular and extracellular viruses compared to IHC and ISH, which mainly detect intracellular viruses. In addition, one needs to consider the fact that the sensitivity of RT-PCR and proteomics may depend on the sample volume, while ISH and IHC methods detect the virus on a thin (5 μm) tissue section. Thus, the results should be put into the context of these limitations and the use of more than one of these assays is recommended to reach optimal sensitivity and specificity.

In conclusion, all methods proved suitable for the detection of EV in FFPE or frozen samples. The new proteomics technologies offer one of the most attractive alternatives for frozen tissues, being relatively sensitive and providing sequence information about the detected virus. On the other hand, the new non-radioactive ISH methods work well in FFPE samples. Even if IHC and proteomics were relatively sensitive, RT-PCR remains one of the most sensitive methods when frozen or fresh samples are available. Importantly, this effort was launched as part of the collaborative efforts of the JDRF nPOD-V Working Group, and these results are guiding virus analyses of pancreas specimens collected from T1D patients.

Conflict of interest

HH is a minor (<5%) shareholder and a member of the Board in Vactech Ltd., which develops vaccines and diagnostic assays for picornavirus infections.

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None

Ethical approval

Not required

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2016.01.015.

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